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XXI Ciclo

Salmonella spp. in Sardinian slaughter pigs: prevalence, serotypes and genotypic characterization

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INTRODUCTION

Foodborne pathogenic bacteria cause illness and death in a large amount of people each year, at great economic cost and human suffering. Most of the pathogens we are struggling with today are not newcomers. *Salmonella*, *Staphylococcus aureus*, *Bacillus cereus* and *Clostridium botulinum* have been associated with foodborne illness for decades (Borch et al., 2002).

Two major sources of foodborne pathogens in meat and meat products may be identified: the living animal carries pathogenic bacteria, while the processing environment harbours them. The human being is also an important source of pathogenic bacteria, most frequently indirectly by cross contamination. Bacteria originating from the animal may, during slaughter, contaminate the carcass, and subsequently be distributed via cut meat or meat raw materials intended for the further processing into meat products (Borch et al., 2002).

Although *Salmonella enterica* is one of the best studied pathogen, the field still has a long way to go, especially considering that:

- it causes significant morbidity and mortality worldwide;

- it has broad host ranges but, for unknown reasons, infections result in different diseases in different hosts;
- it is able to cause persistent infections, which act as reservoirs for transmission shedding;
- it is increasingly resistant to many antibiotics.

The importance of pig as a source of salmonellosis has been shown in a number of investigations (Swanenburg et al., 2001a, b and c; Lo Fo Wong et al., 2002; Botteldoorn et al., 2003; Zengh et al., 2007 among others). Considering the great importance of *Salmonella* as a worldwide food borne pathogen, the control of this agent in pork is a major aim of the swine industry.

The public health risk of *Salmonella* infection from consumption of contaminated pork depends on multiple factors which can be summarized in: the level of infection in the pig herd (Nollet et al., 2005; Nowak et al., 2007; Zheng et al., 2007); the hygienic procedures during slaughtering (Borch et al., 1996; Swanenburg et al., 2001a and c; Botteldoorn et al., 2003); the meat storage and distribution conditions (Boyen et al., 2008); the handling of undercooked pork by the consumer (Hill et al., 2003).

Although *Salmonella* infection in pigs may result in enteric and fatal systemic disease, infected animals generally carry the pathogen asymptomatically in the tonsils, the intestines and the gut-associated lymphoid tissue (GALT); such carriers are a major reservoir of *Salmonella* and pose an important threat to animal and human health (Boyen et al., 2008). In fact, according to Berends et al. (1996), the entire digestive tract, its contents and only the closely associated lymph nodes (tonsils, mesenteric lymph nodes) have practical relevance as major sources of carcass contamination. These are the most important carriers because may serve as reservoirs for further spread of infection through shedding and may end up as contaminated end products (Lo Fo Wong et al., 2002).

The faecal-oral cycle is considered as the main infection route for *Salmonella* in pigs raised in intensive production systems, but its usual presence in nasal discharges of infected pigs (Schwartz, 1999) and the importance of nasal associated lymphoid tissues (NALT) as invasion sites for *Salmonella* (Fedorka-Cray et al., 1995) makes possible that this pathogen is also transmitted by alternative methods, such as nose-to-nose contact and aerosols (Dahl et al., 1996; Hurd et al., 2001b).

The epidemiology of salmonellosis in pigs has been largely investigated and several direct and indirect transmission routes have been demonstrated (Magistrali et al., 2008). *Salmonella* has been identified in all stages of pork production (Lo Fo Wong et al., 2004), since transmission may occur all along the production chain, directly or indirectly from livestock feed, via on-farm production site, at the slaughterhouse or packing plant, in manufacturing, processing and retailing of food, through catering and food preparation in the home (Lo Fo Wong et al., 2002).This means that efforts to decrease the *Salmonella* burden on society could be targeted at various stages of the production chain. An increasing number of European countries are focusing on the primary pork production phase (Lo Fo Wong et al., 2004).

Even though numerous investigations focusing on the epidemiology of salmonellosis in pigs, there is still a lack of consensus in respect to the major sources of infection or contamination (Magistrali et al., 2008).

As previously said, healthy pigs are often infected with *Salmonella* without showing any symptom of disease, so many farmers do not know that their pigs are infected (Swanenburg et al., 2001b). Pigs entering the slaughterhouse that are carriers of *Salmonella* are the most important source of carcass contamination (van der Wolf et al.,

2001a; Zengh et al., 2007). Moreover, pig carcasses contaminated with *Salmonella* will not be recognized during veterinary inspection after slaughter. To confirm that a carcass is contaminated with *Salmonella*, it is necessary to isolate this pathogen from the carcass (Swanenburg et al., 2001a and c).

In the study carried out by Swanenburg et al. (2001c) in a pig slaughterhouse both on slaughtered pigs samples (blood samples, tonsils, liver swabs, tongue swabs, rectal contents, mesenterial lymph nodes and carcass swabs) and slaughterhouse environment (carcass splitter, polishing equipment, scalding water, hands of personnel and drain water), it was demonstrated that the prevalence of pigs with one or more *Salmonella* positive samples was higher than the prevalence of pigs with a positive serotiter against *Salmonella*. This suggests that many pigs had become contaminated during slaughter by cross contamination, and/or got infected with *Salmonella* just before slaughter.

In order to be able to estimate the number of infected animals entering the slaughterhouse and estimate the size of the *Salmonella* "problem" in pig herds, the population and herd level prevalence of *Salmonella* have to be investigated (van der Wolf et al., 2001b): the determination of the *Salmonella* status of pig herds is part of monitoring and intervention programmes to reduce the contamination of pork and it is

necessary to direct interventions at high prevalence herds; culturing faecal samples for *Salmonella* is a useful tool to determine current infections in a pig herd (Lo Fo Wong et al., 2004).

To design an adequate intervention strategy for *Salmonella* contamination in pigs and pork, risk factors all along the production chain need to be investigated.

In the present study we will report the results of a survey carried out with the aim to evaluate *Salmonella* prevalence and serotypes at pig slaughterhouses and on the farm and to investigate *Salmonella* routes by phenotypic and molecular methods.

The experimental study will be preceded by a review about: a brief description of the pig sector in Europe and in Italy; the new EU approach to the control of zoonosis; the characteristics of *Salmonella*, its detection and typing methods, pathogenesis and epidemiology; the official surveillance systems and the risk factors analysis along the pig production chain.

PORK PRODUCTION IN THE EU AND ITALY

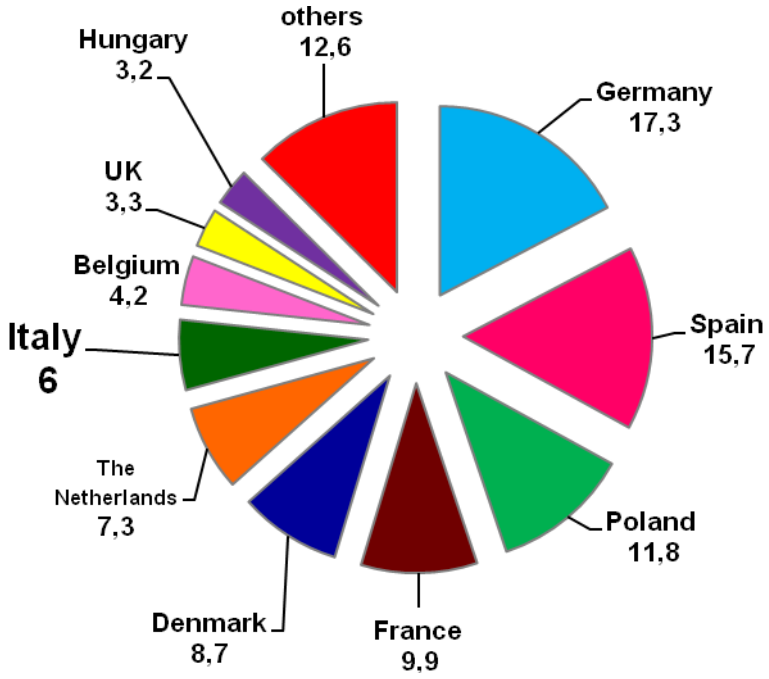
Analysis of the regional contribution to the world's pork production reveals that Europe was until 1980 the leading pork production region in the world. The fast growth of pork production in Asia in the last three decades (the relative increase was 534%) has pushed Europe to the second position in terms of yearly produced tonnes (t) of pork (EFSA 2006).

Pork production shows a great variety of organisational structures and farm size patterns. The organisational pattern ranges from small independent farmers who mainly produce for home consumption and local markets, to vertically integrated agribusiness companies which sell their products on national and international markets. A detailed analysis of the regional pattern shows that the centres of pig production are in most cases closely linked to large and very large pig farms. In addition, the availability of feed, either from domestic production or from imports, plays an important role in the development of regions with intensive pig production.

After China, the EU is the second largest pork producing region in the world. Its pork production in 2005 amounts to about 21 million tonnes, with 17.8 from the EU-15 States and 3.2 million tonnes from the new MS. Of the new EU members, Poland has by

far the largest pork production, with about 2 million tonnes of pork per year (EFSA 2006).

Pig stocks (% of total) in the EU Member States (2004)



Source: FAO database

According to estimations conducted by the German Central Market and Price Reporting System (ZMP), the average per capita pork consumption in the EU-25 was 44.3 kg in 2003. In that year, Italy had a per capita pork consumption between 30.0 and 39.9 kg.

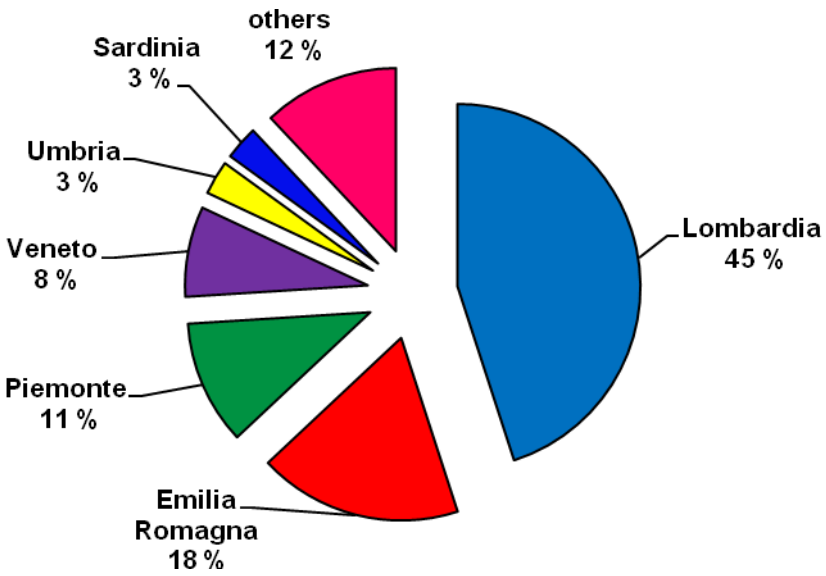
Due to differences both in the production quantities and the consumption habits, there is a considerable amount of pig and pork trading between the EU-25 Member States. Major "exporting" countries (that is, EU-internal trade and not true export) are Denmark, The Netherlands, Belgium, France, Germany and increasingly Spain; major

"importing" countries are Greece, the UK, Italy and the new MS other than Hungary, Poland and Estonia.

The major exposure of consumers to health hazards is likely associated with fresh pork; either directly through consuming raw or undercooked pork, or indirectly through cross-contamination of other food items during meal preparation.

The pig production sector in Italy represents the 17% of the national livestock production (Cossu, 2007). In 2007, as in the previous year, there were nearly 9.3 million swine heads (ISTAT). During the same year, 13.6 millions of pigs have been slaughtered, representing an increase of 1.6 % respect to 2006.

Italian swine production in 2007 (source: ISTAT)



In Sardinia, according to the data from Sardinian Regional Health Authority, in 2007 there were 238,609 swine heads, 70 % of which were sows.

Pig breeding in Sardinia is characterized by different farms:

1. Small farms, in which a few number of swine is reared for home consumption;
2. Small farms complementary to sheep herds;
3. Farms where herds are extensively reared in public pastures, normally located in marginal zones.
4. Indoor intensive farms, normally placed in good geographical zones, with a high number of animals, predominantly raised for production of meat to retail.

The most common swine farm in Sardinia is the semi-extensive. There are 17,920 farms, with an average number of 15.1 swine heads per farm, placed most on the hills but also on mountains and on the plain. The most part of the farms are placed in the Sassari district (about 5,000), followed by Oristano, Ogliastra, Nuoro, Cagliari, Olbia-Tempio, Carbonia-Iglesias and Medio Campidano.

In the centre of the region, outdoor farms are more common than indoor farms.

The swine production is oriented especially to sucking pig production, 5-6 kg weight.

Therefore, the most part of the farms rear sucking pigs (6-10 kg), a very small part store pigs (90-110 kg) and just in very few parts of the inland fattening pigs (140-160 kg) are reared.

In Sardinia there are 74 slaughterhouses.

Slaughtered pigs in 2005 in Sardinia

PIG CATEGORY	NUMBER OF SLAUGHTERED ANIMALS
Sucking pigs	167,984
Store pigs	128,684
Finishing pigs	6,057

Source: Sardinian Regional Health Authority

The average per capita pork consumption is about 32 kg, 35 % of which is represented by pig products thereof. Sardinia only produces 50% of its pork consumption which represents a very low self sufficiency rate. As a consequence there is the need to import pig meat and pig products thereof.

FOODBORNE MICROBIOLOGICAL RISKS: ZOONOSES SURVEILLANCE ACCORDING TO EU LEGISLATION

The data about *Salmonella* in pig meat and products thereof reported by "The Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents, Antimicrobial Resistance and Foodborne Outbreaks in the European Union in 2006", published by the European Food Safety Authority in 2007, show that *Salmonella* was found in moderate proportions of pig meat, up to 13%. Data reported by countries that apply control programmes have showed very low levels of *Salmonella* contamination in fresh pig meat.

As regards to the *Salmonella* occurrence in non ready-to-eat products of pig meat origin, the overall prevalence was included between 0 and 4.8%, and the highest proportion of positive samples was reported by Italy in minced meat.

As regards to ready-to-eat meat, meat preparation and product samples the overall prevalence was included between 0 and 3.4%, with the highest proportion reported from Italy.

The European Food Safety Authority EFSA Summary Report has the aim to publish the data on zoonoses, antimicrobial resistance and food-borne outbreaks collected from the

Member States in accordance with Directive 2003/99/EC as a result of the application of monitoring systems.

The most useful concept for protecting consumer health is to prevent *Salmonella* from entering the food chain.

The European Food Safety Authority (EFSA) was established and funded by the European Community as an independent agency in 2002 following a series of food scares that caused the European public to voice concerns about food safety and the ability of regulatory authorities to fully protect consumers.

EFSA is responsible for examining the data on zoonoses, antimicrobial resistance and food-borne outbreaks collected from the Member States in accordance with Directive 2003/99/EC and for preparing the Community Summary Report from the results.

Zoonoses are defined by the World Health Organisation as "diseases and infections which are naturally transmitted between vertebrate animals and man". Zoonoses are transmitted directly or through ingestion of contaminated foodstuffs and may be bacterial, viral, or parasitic, or may involve unconventional agents (BSE); more than 200 diseases are classified as zoonoses.

The production and consumption of food is central to any society, and has economic, social and, in many cases, environmental consequences. As a consequence, zoonoses transmissible through food may cause human suffering, as well as economic losses to food production and the food industry; zoonoses transmitted through sources other than food, especially from wild animals and pet animal populations, are also matter of concern.

In order to prevent zoonoses from occurring it is important to identify which animals and foodstuffs are the main sources of infections; for this purpose information is collected and analysed from all EU MS to be used to improve control measures aimed to protect human health.

Assuring that the EU has the highest standards of food safety is a key policy priority for the Commission. The European Union's food policy must be built around high food safety standards, which serve to protect and promote the health of the consumer.

EU legislation for the monitoring of zoonoses and zoonotic agents is regulate by the **Directive 2003/99/CE** of 17 November 2003 (amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC).

The objectives of this directive are:

- monitoring of zoonoses and zoonotic agents;
- monitoring of related antimicrobial resistance;
- epidemiological investigation of food-borne outbreaks;
- exchange of information related to zoonoses and zoonotic agents.

"...monitoring should take place on a harmonised basis. This would make it possible to evaluate trends and sources of zoonoses and zoonotic agents within the Community. Priority should be given to those zoonoses posing the greatest risk to human health. However, the monitoring should also facilitate the detection of emerging or newly emerging zoonotic diseases and new strains of zoonotic organisms...".

"...in addition to general monitoring, specific needs may be recognised which may necessitate the establishment of coordinate monitoring programmes. Attention should be paid in particular to zoonoses listed in Annex I to regulation (EC) No 2160/2003...".

"...it is necessary to improve the existing monitoring and data collection systems established by Directive 92/117/EEC. Simultaneously, Regulation (EC) No 2160/2003 of the European Parliament and of the Council of 17 November 2003 on the control of *Salmonella* and other specified food-borne zoonotic agents will replace the specific

control measures established by Directive 92/117/EEC. Directive 92/117/EEC should therefore be repealed…".

Regulation (EC) n.2160/2003 of 17 November 2003 regulate the control of *Salmonella* and other specified food-borne zoonotic agents.

The objectives of the Regulation (EC) n.2160/2003 are:

- the adoption of targets for the reduction of the prevalence of specified zoonoses in animal populations at the level of primary production and, where appropriate for the zoonosis or zoonotic agent concerned, at other stages of the food chain, including in food and feed;
- the approval of specific control programmes established by Member States and food and feed business operators;
- the adoption of specific rules concerning certain control methods applied in the reduction of the prevalence of zoonoses and zoonotic agents;
- the adoption of rules concerning intra-Community trade and imports from third countries of certain animals and products thereof.

Referred to the Directive 92/117/EEC, repealed by the Directive 2003/99/EC, it says:"...the results of the data collection systems show that certain zoonotic agents,

namely *Salmonella* spp. and *Campylobacter* spp., cause the majority of cases of zoonoses in humans. There seems to be a decreasing trend of human cases of salmonellosis, in particular due to *Salmonella enteridits* and *Salmonella typhimurium*, thus reflecting the success of related control measures taken in the Community. Nevertheless, it is assumed that many cases remain unreported and therefore the data collected do not necessarily give the full picture of the situation...".

MS will have to prepare compulsory control programmes for *Salmonella* serovars with public health relevance (prevalence, rapid or recent diffusion, virulence). Such programmes must be aimed to reach precise and measurable targets of prevalence reduction, defined by the Commission. "…the targets should be established for zoonoses and zoonotic agents in animal populations taking account, in particular, of their frequency and epidemiological trends in animal and human populations, feed and food, their gravity for humans, their potential economic consequences, scientific advice and the existence of appropriate measures to reduce their prevalence. Targets may be established in respect of other parts of the food chain, where necessary…".

Monitoring programmes harmonized at the EU level, to define infection prevalence and subsequently establish targets of reduction; such programmes must allow to follow

prevalence trends during time, in order to verify targets achievement; once targets are established, the Member States will have to prepare control programmes, to be submitted to the Commission for approval.

After the control programmes have been approved, food business operators must have samples taken and analysed to test for the zoonoses and zoonotic agents, respecting minimum sampling requirements; as regards to the control of *Salmonella* in pigs, the animal populations to be controlled are breeding and slaughter pigs: animals leaving for slaughter or carcases at the slaughterhouse.

The Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs "…lays down the microbiological criteria for certain micro-organisms and the implementing rules to be complied with by food business operators when implementing the general and specific hygiene measures referred to in Article 4 of Regulation (EC) No 852/2004". This Regulation laid down food safety criteria for certain important foodborne bacteria, their toxins and metabolites, such as *Salmonella*, *Listeria monocytogenes*, *Enterobacter sakazakii*, staphylococcal enterotoxins and histamine in specific foodstuffs.

Commission Regulation (EC) No 2073/2005 on microbiological criteria was amended by **Regulation (EC) 1441/2007** of 5 December 2007 with regard to:

- criteria on Enterobacteriaceae and *Salmonella* in dried follow-on formulae and *Bacillus cereus* in dried infant formulae;
- the reference method for the staphylococcal enterotoxin detection;
- specifies the *Salmonella* sampling rules for carcasses of cattle, pig, sheep, goats and horses.

As regards to food safety criteria, the Regulation establishes that *Salmonella* must be absent in every food category; these rules must be applied when the products are placed on the market during their shelf-life (with the analytical reference method EN/ISO 6579). As regards to process hygiene criteria referred to carcases of pigs, criterion must be applied after dressing but before chilling and *Salmonella* must be absent in the area tested per carcass (analytical reference method ISO 6579); in case of unsatisfactory results the actions to be taken consist in improvements in slaughter hygiene and review of process controls, origin of animals and of biosecurity measures in the farms of origin. However, the Regulation admits the possibility to detect 5/50 (c/n) positive samples to

*Salmonella***.** The *c* value is subject to review in order to take into account the progress made in reducing the *Salmonella* prevalence. Member States or regions having low *Salmonella* prevalence may use lower c values even before the review.

SURVEILLANCE OF ZOONOSES IN EUROPE

FRAMEWORK OF REPORTING

The Community system for monitoring and collection of information on zoonoses is based on the Zoonoses Directive 2003/99/EC, which obligates the European Union Member States to collect relevant and comparable data of: 1) zoonoses; 2) zoonotic agents; 3) antimicrobial resistance; 4) foodborne outbreaks.

In addition, Member States shall assess trends and sources of these agents and outbreaks in their territory, and transmit to the European Commission, every year, a report covering the data collected. The European Food Safety Authority (EFSA) is assigned the tasks of examining the data collected and publishing the Community Summary Report.

The Decision 2119/98/EC on setting up a network for the epidemiological surveillance and control of communicable diseases in the Community, established the data collection on human communicable diseases from the Member States. The Decisions foresee that data from the networks shall be used in the Community Summary Report on Zoonoses. In such report the data related to the occurrence of zoonotic agents in animals,

foodstuffs and feed as well as to antimicrobial resistance in these agents are collected in the framework of Directive 2003/99/EC. This applies also to information of foodborne outbreaks.

Since 2005, the European Centre for Disease Prevention and Control (ECDC) has provided the data on zoonotic infections in humans, as well as the analyses of these, for the Community Summary Report. The data used for analysis were derived from several disease networks: the new European Surveillance System (TESSy); and two Dedicated Surveillance Networks (DSN): Enter-Net for *Salmonella* and *E.coli* verotoxigenic (VTEC) surveillance, and Euro-TB for tuberculosis surveillance.

ENTER-NET (Enteric Pathogen Network) is the european surveillance system for *Salmonella* and *E.coli* (VTEC) infections in humans. It is coordinated by the Health Protection Agency (HPA), comprises 23 European countries and collaborate with non-European countries (Japan, Canada, Australia, South Africa). Italy is represented in the network by the Istituto Superiore di Sanità (ISS). In 2002 the Enter-vet network has been established for the surveillance of *Salmonella* veterinary infections with the cooperation of the Istituti Zooprofilattici Sperimentali (IZS), coordinated by the Italian *Salmonella* reference laboratory (IZS Venezie, Padua). The integrated networks Enter-

Net and Enter-Vet collect every year the data regarding *Salmonella* human and non humans (food, animals, environment) cases.

The Rapid Alert System for Food and Feed (RASFF) is a system which has been in place since 1979 and whose purpose is to provide the control authorities with an effective tool for exchange of information on measures taken to ensure food safety.

The legal basis of the RASFF is the Regulation EC/178/2002 that laid down the general principles and requirements of food law, established the European Food Safety Authority and lad down the procedures in matters of food safety.

The Annual Report on the RASFF provides useful data on the number of notifications received every year, as well as details on the origin of the notifications, the products and countries involved, and the identified risks. It also details the follow-up actions carried out in response to various food safety problems.

To assist the members of the network, information is classified under two different headings:

• Alert notifications: are sent when the food or feed presenting the risk is on the market and when immediate action is required. Alerts are triggered by the Member State that detects the problem and has initiated the relevant measures, such as withdrawal/recall.

The notification aims to give all the members of the network the information to verify whether the concerned product is on their market, so that they also can take the necessary measures. The Member States have their own mechanisms to carry out such actions, including the provision of detailed information through the media if necessary.

• Information notifications: concern a food or feed for which a risk has been identified, but for which the other members of the network do not have to take immediate action, because the product has not reached their market. These notifications mostly concern food and feed consignments that have been tested and rejected at the external borders of the EU.

SALMONELLA: MICROORGANISM CHARACTERISTICS

The role of *Salmonella* in food-borne disease was first documented in the late 1800s, although association with human clinical disease, in the form of typhoid, dates back to the beginning of that century. In 1885, an organism designated *Bacillus cholera-suis* was isolated by a veterinary pathologist, D.E. Salmon, from pigs suffering hog cholera. Other similar organisms were isolated from outbreaks of food-borne disease and infected animals. To accommodate these organisms, the genus *Salmonella* was created by Lignières in 1900, in honour of Salmon (Cox, 2000).

Salmonella are Gram negative rods, 0.7-1.5x2.0-5.0 µm, within the family of *Enterobacteriaceae*. Members of this genus are usually motile by peritrichous flagellation except serovar Gallinarum and Pullorum which are always nonmotile, with lack of flagella. Nonmotile variants can also arise as a result of a faulty assembly of flagellar subunits or deficiencies in the motor functions of these appendages (D'Aoust, 2000).

Salmonella are facultatively anaerobic. Most are aerogenic. However, serovar Typhi, an important exception, never produces gas. Anaerogenic variants of normally gas-

producing *Salmonella* serovars may occur; this is particularly common with serovar Dublin. Colonies are generally 2-4 mm in diameter but certain serovars, such as serovar *Abortusovis*, may form unusually small colonies (~1 mm diameter), whereas most types form larger colonies (2-4 mm) (Popoff and Le Minor, 2005).

Salmonellae grow optimally at 35 to 37 °C and catabolize a variety of carbohydrates into acids and gas use citrate as the sole carbon source, produce H_2S, and decarboxylate lysine and ornithine to cadaverine and putrescine respectively. These microorganisms are oxidase-negative and catalase-positive with 50 to 53 mole % guanine plus cytosine (G+C) DNA content (D'Aoust, 2000).

Historically, an isolate from selective plating media was most likely to be confirmed as *Salmonella* if it catabolised glucose and lysine but it failed to metabolise lactose, sucrose and urea. The reliability of these diagnostic traits is being undermined by the widespread exchange of genetic elements between compatible bacterial strains in the environment. Atypical *Salmonella* biotypes that cannot decarboxylate lysine or that readily use lactose, sucrose and urea have been isolated (D'Aoust, 2000).

PHYSIOLOGY

A range of environmental conditions affects the growth, death or survival of *Salmonella*.

TEMPERATURE

Salmonellae can growth within the range 2-54 °C, although growth below 7°C has been observed only in bacteriological media (not in foods), while growth above 48°C is confined to mutants or tempered strains. The optimum temperature for growth is 37 °C, which is not surprisingly given that the natural ecology of most *Salmonella* strains of concern to public health is the gastrointestinal tract of warm-blooded animals. Above the maximum growth temperature, salmonellae die quickly and are, in general, readily destroyed by mild heat processes, such as pasteurization. However, susceptibility varies with strains. Exposure to adverse conditions, including sub lethal temperatures and extremes of pH, increases resistance.

Salmonella survive quiet well in at low temperatures. Although the time varies with substrate and the influence of factors such as pH and a_w, strains may survive for days to weeks at chill temperatures. During freezing, a population of *Salmonella* will be reduced in inverse proportion to the rate of freezing, further influenced by the degree of

protection afforded by the matrix in which the organism is held and the physiological status of the cells. Log-phase cells are more susceptible to damage. After freezing, a population of *Salmonella* undergoes a slow decline which is inversely proportional to the storage temperature. In a protective matrix, and under commercial freezing conditions, *Salmonella* may survive for months or years (Cox, 2000).

pH

The optimum pH for growth of *Salmonella* is within the range 6.5-7.5 pH. Strains grow at pH values up to 9.5 and down to 4.05. While growth occurs down to or close to the minimum pH with non-volatile organic acids such as citric acid, or mineral acids such as hydrochloric acid, growth stops at higher pH values when volatile fatty acids (VFAs) are used. The inhibitory effect of VFAs is inversely proportional to chain length, and increases under anaerobic conditions, presumably due to a decrease in available energy (ATP) and a consequent decrease in ability to remove the acids from the intracellular environment.

Increasing temperature increases sensitivity to low pH, as does the presence of food additives such as salt or nitrite.

Tolerance or adaption to low pH is significant with respect to virulence, increasing the likelihood of surviving gastric acidity, or the acidic intracellular environment of phagocytic cells (Cox, 2000).

WATER ACTIVITY

Salmonella grows at a_w values between 0.999 and 0.945 in laboratory media, down to 0.93 in foods, with an optimum of 0.995. While there is no growth below 0.93, *Salmonella* survives; the time of survival increases as a_w decreases. Salt (NaCl), used as a solute to lower a_w, and as a preservative in foods, is inhibitory towards *Salmonella* at concentrations of 3-4% tolerance increases with temperature between 10 and 30 °C (Cox, 2000).

VIRULENCE FACTORS

LYPOPOLYSACCHARIDE

Variation in the amount of the LPS produced, the length of O side chains, and the degree of glycosilation affect virulence which is enhanced when the former properties are increased. Long side chains sterically hinder the ability of components of the complement cascade system to bind to the surface of the *Salmonella* cell, preventing lysis (Cox, 2000).

FIMBRIAE

When moving ahead in the host gastrointestinal tract, the invading salmonellae face, after acidic stomach, favourable pH and nutritional conditions in the small intestine and form colonies, eventually biofilms on host cells in mucosal membranes. Salmonellae rapidly invade the gut epithelium in humans. They possess several surface structures, fimbriae, for attachment. The members of the genus *Salmonella* have genes coding at least 12 different fimbrial types, many of which have been associated to the bacterial virulence. The most intensively studied fimbrial structures are the enterobacterial type 1

fimbriae, which mediate the mannose-sensitive binding of the bacterial cells to the target cells (Hakalehto et al., 2007).

TOXINS

The capacity of salmonellae to induce intestinal fluid secretion is, in part, attributed to the production of a protein enterotoxin (Chopra et al., 1999) which is produced by many strains of *Salmonella*. Such enterotoxin appears to be structurally similar to cholera toxin, consisting of A and B subunits that act respectively to stimulate host cell adenylate cyclase and produce a pore through which the former enters. Increased levels of cellular cyclic AMP (cAMP) lead to a net massive increase in concentration of sodium and chloride ions and a consequent accumulation of fluid in the intestine lumen. *Salmonella* also produces a membrane-bound proteinaceous cytotoxin, which is serologically and genetically distinct from Shiga toxins of *Shigella* and *E.coli*. The toxin, which may be released intracellularly as a consequence of limited bacterial lysis, inhibits protein synthesis, leading to host cell lysis and dissemination of the bacterium. Host cell lysis may also result from chelation of divalent cations by the toxin, causing disruption of host cell membranes (Cox, 2000).

SIDEROPHORES

Like many other members of Enterobacteriaceae, *Salmonella* produces two types of sequestering molecules, or siderophores, to acquire iron which is critical to survival and growth. The first is a high affinity siderophore known as enterochelin or enterobactin, while the second is called aerobactin. Both these molecules sequester ferric ions from the environment (intestinal lumen, serum), and after binding to an outer membrane protein is translocated to the cytoplasm, where Fe^{+++} is reduced to Fe^{++}, which is released from the siderophore. Strains producing enterochelin are generally more virulent than those producing aerobactin (Cox, 2005).

OTHER CHROMOSOMALLY ENCODED FACTORS

A series of genes, the products or functions of which have not been fully characterized, occur within large gene loci, termed pathogenicity islands. A series of 15 genes, the *inv* region, occurs within such island, and is necessary for epithelial cell invasion. Gene products are responsible for early stages of cell engulfment, including epithelial cell membrane ruffling and assembly and translocation to the bacterial cell surface of attachment appendages the inv genes are expressed during late logarithmic or early

stationary phase under condition of high osmolarity and low oxygen tension, conditions encountered in many internal body sites.

Other regions of the chromosome encode factors necessary for intracellular survival: the *oxyR* locus encodes proteins protective against the toxic oxygen products in macrophages, while the *phoP/phoQ* regulatory system is required for expression of factors permitting survival within phagocytic cells (Cox, 2000).

PLASMIDS

Most of the serovars of *Salmonella enterica* subspecies do not possess any plasmid. Serovars such as Typhi, Paratyphi, Hadar, Infantis and most of the exotic serovars are usually free of any plasmid. This is not valid for the serovars which are frequently associated with infections of humans and farm animals including choleraesuis, dublin, enteritidis, pullorum and typhimurium. In strains of these serovars it is quite difficult to find a field strain which would be free of any plasmid (Rychlik et al., 2006). Such serovars harbour large serovar-specific plasmids and, although these plasmids vary considerably in size, incompatibility group and overall omology, they contain an essentially identical piece of DNA, known as the *Salmonella* plasmid virulence or *spv*

region. The region contains at least five genes, *spvRABCD*. For any serovar, the role in virulence played by the *spv* gene products varies with host. For example, the plasmid of serovar enteritidis affects invasion in cattle and mice, but not chickens (Cox, 2000). Interestingly, some host-adapted serovars such as Typhi and gastroenteric serovars with a predisposition to extraintestinal infection, such as Virchow, do not harbour virulence plasmids; in some cases the homologous virulence genes have been located within the chromosome of these serovars (Cox, 2000).

Plasmids in *Salmonella* control medically important properties including virulence factors, resistance to heavy metals, antibiotics, phages or utilisation of alternative carbon sources. Since plasmids code for genes dispensable for the functioning of the host cell, they represent genetic information under a lower selection pressure which can be subjected to an accelerated evolution. Therefore, acquisition of a plasmid allows its host to adapt to a changing environment more readily (Rychlik et al., 2006).

Plasmid classification

Plasmids are most frequently classified into incompatibility groups according to their mode of replication and maintenance in a bacterial cell. Plasmids of different modes of

replication are able to reside in the same bacterial cell while two different plasmids exploiting the same replication machinery are mutually incompatible and unable to persist in the same cell for a prolonged period (Rychlik et al., 2006).

OTHER VIRULENCE FACTORS

Unique *Salmonella* virulence traits are thought to have been acquired by horizontal gene transfer and integration into the bacterial chromosome. An example is the discrete chromosomal virulence gene insertions termed *Salmonella* Pathogenicity Islands (SPI). Five SPIs have been identified and are common across numerous serotypes. These determinants, along with virulence traits encoded on plasmids common to many strains, are responsible for the specific interactions that work together to allow *Salmonella* to become host adapted and a successful pathogen (Cox, 2000).

TAXONOMIC CLASSIFICATION

EVOLUTION OF *SALMONELLA* NOMENCLATURE

Several schemes based on biochemical characteristics, DNA homology, and enzyme electrophoretic patterns have been used for taxonomic classification of salmonellae (D'Aoust, 2000).

In 1966, Kauffmann used determinant biochemical traits to separate the *Salmonella* group into four subgenera (I to IV) with each serovar given species status. A fifth subgenus (V) was subsequently recognized. The main characteristics of each subgenus included the ability to use certain substrates:

- Subgenus I: inositol;
- Subgenus II: dulcitol and malonate;
- Subgenus III: malonate and o-nitrophenyl-ß-D-galactopyranoside (ONPG);
- Subgenus IV: growth in Moeller KCN Broth Base (supplemented with a solution of potassium cyanide) and failure to metabolize dulcitol and malonate;
- Subgenus V: catabolism of ONPG and growth in KCN.

In 1972, Ewing proposed another taxonomic scheme that recognized only three species within the *Salmonella* group. These includes *S.typhi* (single serovar), *S.cholearesuis* (single serovar) and *S.enteritidis*, which encompassed all of the remaining serovars. Members of the Arizona group were assigned to a distinct Arizona genus. This scheme was structured on the differential utilization of 15 substrates and production of hydrogen sulphide gas.

A third taxonomic scheme based on phenotypic and DNA homology characteristics recognized *S.choleraesuis* as the sole species subdivided into seven subspecies including subspecies *choleraesuis*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *bongori*, and *indica*. In 1987, the latter scheme was modified to recognize *S.enterica* as the sole species consisting of the seven foregoing subspecies *choleraesuis* to subspecies *enterica*. The type strain was also changed from *S.choleraesuis* to *S.enterica* subspecies enterica serovar Typhimurium LT 2. In 1989, the subspecies bongori was elevated to species level based on discriminating multilocus enzyme electrophoretic patterns.

The *Salmonella* genus presently consists of two species: *S.enterica* including six subspecies (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*) and *S. bongori* (D'Aoust, 2000).

METHODS FOR DETECTION OF *SALMONELLA*

There is a variety of conventional culture and rapid methods in use for the detection of *Salmonella* in foods.

CULTURE METHODS

Most of the culture methods consists in five steps:

1. Pre-enrichment;
2. Selective enrichment;
3. Selective/differential plating;
4. Biochemical testing;
5. Serological testing.

The physiological state of *Salmonella* has a profound effect on culturability. In a food sample *Salmonella*, if present, will be in low numbers and often in a poor physiological state, suffering injury due to processes, such as chilling, freezing, heating or extremes of pH. Nevertheless, such cells are still capable of recovery after ingestion, potentially causing disease, and thus must be detected. To aid recovery of *Salmonella* and facilitate the detection process, food samples are subjected to non-selective liquid pre-enrichment

(resuscitation). This is followed by selective liquid enrichment, permitting further growth of the now vegetative *Salmonella*, while suppressing the background flora that develops during resuscitation. Finally, the selective enrichment are plated, both to suppress the growth of competitors and to allow for the isolation of discrete suspect colonies; then, any isolates characterized.

Most *Salmonella* exhibit a common pattern of biochemical reactions and physiological traits, many of which are exploited in cultural methods for detection. However, some strains may display some or rarely more atypical reactions/traits, including fermentation of disaccharides such as lactose and sucrose, failure to produce hydrogen sulphide, lack of lysine decarboxylation or lack of motility. In the case of such strains, cultural detection may fail. Atypical strains are rare in relation to the many thousands isolated annually, occurring at an incidence of less than 0.1% for any given trait. However, the incidence of atypical strains in relation to a specific food matrix may be much higher, due to selective pressure: an example is lactose-positive strains in dairy products (Cox, 2000).

NONCULTURAL METHODS

Although *Salmonella* is a non-fastidious microorganism and shows fast growth with microbiological culture, up to 72 h or even more is required to culture and type *Salmonella* isolates. Since PCR and various PCR-based technologies provide fast results and a high degree of specificity, they constitute a valuable tool in microbiological diagnostics. The multiplex-PCR applied gives best results, with number of positive results similar to those obtained by bacteriological method, and reduce the time needed for the detection of *Salmonella* (Cortez et al., 2006). Other non-cultural techniques, such as latex agglutination and enzyme-linked immunoassorbent assay, can be use for the detection of *Salmonella,* including many atypical strains. Such methods can be highly accurate (some are >98% in agreement with a reference cultural method), but normally they are not considered definitive because they usually do not produce an isolate. Rapid methods that exhibit both high specificity and high sensitivity can be used as a screening tool when they are performed in tandem with the culture method (especially when the rapid method and the culture method share the same pre-enrichment strategy). In cases where a validated rapid method does not detect *Salmonella* in a pre-enrichment that it shares with the culture method, the culture

method can be discontinued. In such a case, the rapid method has demonstrated, to a high degree of probability, that *Salmonella* are not present in the test sample and no further work need to be done. Thus, rapid methods can be used to save time and resources (Cox, 2000).

TYPIZATION OF *SALMONELLA*

SEROTYPING AND PHAGE TYPING

The primary method used for characterizing members of the genus *Salmonella* is serotyping.

This technique allows to differentiate strains, which are epidemiologically the smallest bacterial unit from which isolates share the same phenotypic and genotypic traits. Strains are divided into serogroups, based on differences in epitopes of lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria and is designated the O or somatic antigen for serological purposes. Within serogroups, strains are further differentiated into serovars, based on variation in flagellins or H antigens, the subunit proteins of flagella. Some serovars are easily defined with respect to H antigens as they produce only one form, or phase; these serovars are termed monophasic. However, most serovars, termed diphasic, are capable of producing two distinct forms of flagella. Some strains are genotypically triphasic, capable of producing a third phase H antigen, which may encoded chromosomally or more often extrachromosomally (plasmid borne), although phenotypically these strains

appear diphasic. An alternative H antigen produced in Phase 1 is referred to as a R-phase H antigen (Cox, 2000).

The serotype is deduced from the specific pattern of agglutination reactions using the Kauffmann-White classification scheme The Kauffmann–White scheme recognizes 46 O serogroups and 114 H antigens resulting in 2523 characterized serotypes (Popoff and Le Minor, 2001).

No fewer than 2422 serotypes are recognized within the genus and the number continue to increase.

On the basis of the level of host restriction, *Salmonella* serotypes can also be classified as:

1) host-restricted serotypes: serotypes capable of causing a typhoid-like disease in a single host species: (e.g. *S. typhi* in humans);
2) host adapted serotypes: serotypes associated with one host-species, but also able to cause disease in other hosts (e.g. *S. choleraesuis* and *S. typhisuis* in swine);
3) broad host range serotypes: vast majority of the remaining serotypes which rarely produce systemic infections but are able to colonize the alimentary tract of a wide range of animals (e.g. *S.typhimurim* and *S.derby*).

Serotyping is a reliable, epidemiologically congruent, well established methodology for *Salmonella* typing, but is rather time consuming and requires a high number of specific antisera.

Moreover, serotyping is not efficient enough when trying to track the source of common source outbreaks in the most frequently isolated serotypes.

To further discriminate within serotypes, phage typing is the primary sub-typing technique (Torpdahl et al., 2005). Strains within a particular *Salmonella* serotype may be differentiated into a number of phage-types by their patterns of susceptibility to lysis by a series of phages with different specificities. A bacteriophage is a virus which specifically attacks bacteria. Bacteriophages may have a lytic cycle or a lysogenic cycle, but a few viruses are capable of carrying out both. With lytic phages, bacterial cells are broken open (lysed) and destroyed after immediate replication of the virion. As soon as the cell is destroyed, the new bacteriophages viruses can find new hosts. In contrast, the lysogenic cycle does not result in immediate lysing of the host cell. Those phages able to undergo lysogeny are known as temperate phages. Their viral genome will integrate with host DNA and replicate along with it fairly harmlessly, or may even become established as a plasmid. The virus remains dormant until host conditions deteriorate,

perhaps due to depletion of nutrients, then the endogenous phages (known as prophages) become active. At this point they initiate the reproductive cycle resulting in lysis of the host cell. As the lysogenic cycle allows the host cell to continue to survive and reproduce, the virus is reproduced in all of the cell's offspring. Sometimes prophages may provide benefits to the host bacterium while they are dormant by adding new functions to the bacterial genome in a phenomenon called lysogenic conversion. As regards to *Salmonella*, lysogenization by certain converting phages may produce changes in the O antigenic formulae of salmonellae (Popoff and Le Minor, 2001). Banks of phage have been developed for a number of serotypes including S. *typhimurium* and S. *enteritidis*.

Unfortunately, phage typing frequently fails to discriminate between outbreak-related and unrelated isolates (Torpdahl et al., 2005).

Antimicrobial resistance profiling is another phenotypic method commonly used to investigate diversity among *Salmonella* strains. It is normally performed determining the resistance minimum inhibitory concentration (MIC) break-points by the disk diffusion method or the agar diluition method.

GENOTYPIC CHARACTERIZATION

Epidemiological tracing should be based on the association of independent genotypic and phenotypic characteristics (Cado Bessa et al., 2007).

We have already outlined the importance of the antimicrobial resistance profile; furthermore, genetic characterization of antimicrobial resistance genes as well as their location and diversity, is important in identifying factors involved in resistance, understanding the diversity of MDR strains, identifying genetic linkages among markers and understanding potential transfer mechanisms (Gebreyes and Altier, 2002).

Several DNA-based typing methods have been developed in an attempt to improve the reproducibility and discriminatory ability in typing of *Salmonella*.

Molecular typing methods are useful for defining clonal relationships between strains and provide more epidemiological information about the nature of contamination (Torpdahl et al., 2005).

Each typing technique has tradeoffs, since an all encompassing typing technique

satisfying all the typing needs for *Salmonella* has yet to be developed. Subsequently, selection of the most appropriate molecular typing method is made considering factors like sample size, turn-around time, and resources available to perform the typing (Yan et al., 2003).

1) Restriction digestion based techniques

- Pulsed field gel electrophoresis (PFGE) and other restriction fragment length polymorphism analysis (RFLP) techniques are based upon DNA isolation and restriction fragment analysis (Torpdahl et al., 2005). PFGE separates DNA under conditions of alternating polarity allowing for the resolution of DNA fragments nearly 20 times larger than those separated by traditional agarose gel electrophoresis. When used in conjunction with rare cutting restriction enzymes, PFGE profiles provide a DNA fingerprint of the bacterial genome. In addition to PFGE, RFLP analysis can be performed using a frequent cutting enzyme to restrict the DNA. Because a large number of fragments are generated, electrophoresis is typically followed by southern blotting using probes for repeated DNA elements. Common blotting targets include the ribosomal RNA gene sequences (ribotyping) and different insertion elements in the bacterial genome (insertion sequence (IS) typing) (Yan et al., 2003). PFGE has become

a method of choice to elucidate the relationship of *Salmonella* strains and to assess the distribution of clonal strains (Giovannacci et al., 2001; Cado Bessa et al., 2007); it is widely used and has been shown to be very effective for both epidemiological surveillance and outbreak investigations in several serotypes of *Salmonella*, including Enteritidis (Powell et al., 1994), and Typhimurium (Olsen et al., 1997).

2) Amplification based techniques

- Amplified fragment length polymorphism (AFLP) couples restriction digest analysis and PCR amplification to determine the relatedness of bacterial strains. Following digestion with one or more enzymes, linker DNAs are ligated to the free DNA ends. These linkers serve as targets for PCR primers to bind and allow selective amplification of the restriction fragments. The amplified fragments are subjected to electrophoresis, and characteristic separation profiles are generated and compared to other strains (Yan et al., 2003).

AFLP has been shown to be a useful tool in epidemiological surveillance and outbreak investigations of *S. enteritidis* (Scott et al., 2001) but have also shown limitations in *S. typhimurium* (Lawson et al., 2004). Several investigations have been comparing the

discriminatory level of PFGE and AFLP and the discriminatory power of the two techniques seems to be very similar although dependant on the serotype investigated (Gebreyes et al., 2005; Torpdahl et al., 2005).

- Other PCR-based typing methods rely on amplification profiles, without DNA restriction, to separate bacterial isolates. Two of these techniques are RAPD-PCR and AP-PCR, which are similar methods of PCR subtyping. These techniques use PCR primers of random sequence. When two of the primers bind in close enough proximity to one another, they amplify the intervening portion of the genome, creating variably sized amplicons (Yan et al., 2003).

3) Nucleotide sequencing based techniques

- Multilocus sequence typing (MLST) is based on the variability in the sequence of particular genes of different strains of bacteria, due to mutation or recombination events, that can be utilized to determine the relatedness of bacteria. With MLST, multiple housekeeping genes from an isolate are sequenced. For each gene, the different sequences present within a bacterial species are assigned as distinct alleles. For each isolate, the alleles at each locus define the allelic profile or sequence type, and the

results compared to the sequences of other strains are used to determine nucleotide base changes between isolates. Nucleotide differences in the individual genes are combined and used to generate the multilocus sequence type, which is subsequently used to determine the relatedness of different strains (Yan et al., 2003).

Plasmid profiling is another simple and inexpensive technique which is useful as a means of subtyping PFGE types. It is based on the agarose gel electrophoresis of the plasmidic DNA which is normally obtained with alkaline extraction. It has minimal requirements, gives rapid results (< 1 day possible) and can provide additional information to supplement and assist in interpretation of PFGE data (Schaberg et al., 1981).

CURRENT NOMENCLATURE

Whitin the subgenus system, at the serotype level, *Salmonella* have historically afforded species status. The name of a particular serotype appears in a typical italicized genus-species form (e.g. *S.typhimurium*). Only serovars of subspecies enterica are given names and the serovar name is not afforded species status. Thus, serovar typhimurium is fully described as *S.enterica* subsp. *enterica* serovar *typhimurium*, which is conveniently abbreviated to *S.typhimurium.*

Serovars in the remaining subspecies of *S.enterica* and in *S.bongori* are identified by antigenic formulae, although some of the older and more common serovars of subspecies other than *S.enterica* are still often referred to by name (e.g. S. enterica subsp. salamae serovar 1,4,12,27:b:[e,n,x] is still commonly known as *S.sofia*.

Salmonella serovars were originally named for the disease syndrome in various hosts, examples being serovars typhi, typhimurium, abortusovis and bovismorbificans in humans, mice, sheep, and cattle respectively. Shortly thereafter, nomenclature based on species and syndrome became limiting, and names were assigned according to the first geographical site of isolation (e.g. *adelaide*, *dublin*, *london* etc.) (Cox, 2000).

PATHOGENICITY IN HUMANS

INFECTIOUS DOSE

Infection begins with ingestion of a dose of the bacterium sufficient to broach the first-line host defences and colonize the gastrointestinal tract.

While the typical infectious dose is considered to be in the range of 106-108 cfu, epidemiological evidence from a number of outbreaks has demonstrated that the infectious dose may be substantially less, as little as a few cells (Boyle et al., 2007).

The dose required is influenced by some factors:

- the nature and physiological status of the strain itself: different strains of *Salmonella* possess a diversity of virulence factors which can be brought to bear against the host defences; the number and type of these factors have a profound effect on pathogenesis. The physiological state of the organism, whether in active log phase or the stationary phase, may have an impact on survival, both in the food matrix and upon entry into the host.
- the status of the potential host: the host also influences infectious dose. Even though *Salmonella* infections in humans caused by non-typhoidal serotypes are

usually selflimiting, effective antimicrobial therapy is essential if systemic spread occurs. Systemic spread is frequently seen in individuals with immunodeficiency due to immaturity, senescence, chemotherapy, gastric hypoacidity, pregnancy or antecedent diseases. Together, individuals with these conditions constitute the so-called YOPI (young, old, pregnant, immunodeficient) segment of the population. This group comprises 10–15% of the human population (Boyen et al., 2008). The very young have a poorly developed immune system and low gastric acidity, while the elderly and immunocompromised demonstrate only a weak immune response against infection.

- the matrix in which the strains is ingested: a food matrix high in fat or protein offers significant protection to the organism within it, both in relation to the external environment, and that within the host. Such foods act as a barrier to gastric acidity, and those high in fat are also voided quickly from the stomach, both serving to transport *Salmonella* quickly and without injury to the lower gastrointestinal tract. Additionally, cells present in such a matrix will be in a dormant and thus more resistant physiological state.

DISEASE

From the standpoint of human disease, *Salmonella* serotypes can be divided into three groups that cause distinctive clinical syndromes:

1. Typhoid fever
2. Enteritis
3. Bacteremia

Typhoid (enteric) fever

Typhoid fever is a systemic infection that is caused by *Salmonella* serotypes which are strictly adapted to humans or higher primates, including *Salmonella enterica* serotypes *Typhi*, *Paratyphi A, Paratyphi B* and *Paratyphi C*. The disease is currently rare in the United States and Europe but endemic in Asia, Africa and South America from where it can be imported by foreign travel (Boyle et al., 2007). The microorganism is transmitted by the faeco-oral route, thus the disease is often associated with poor sanitation and hygiene (Cooke et al., 2007).

Over the last few decades, there has been an increase in the number of people travelling to typhoid-endemic countries, and immigrants or refugees from endemic areas may

carry the typhoid bacillus into non-endemic countries (Cooke et al., 2007). In 1987, the incidence of typhoid amongst travellers to developing countries was estimated as 3—30 cases per 100 000 travellers (Steffen et al., 1987).

Fever is the first symptom and manifests after a median incubation period of 5-9 days, depending on the challenge dose; typhoid fever patients are often constipated during the early stages of infections but about one third develop diarrhea subsequent to the onset of fever. Biopsies taken from the upper small intestine as early as three days post experimental infection of volunteers with serotype Typhi reveal diffuse enteritis caused predominantly by a mononuclear leukocyte infiltrate (Cox, 2000).

A significant proportion of typhoid patients become chronic carriers of serovar Typhi, as do many people who have never had a clinical history of typhoid fever. These individuals shed high numbers of bacteria in their stools for long periods (up to a lifetime!) without obvious sings of disease (Boyle et al., 2007).

Enteritis

Enteritis is caused by any of more than 2500 *Salmonella* serotypes, however *S.enterica* serovar *Typhimurium* and *Enteridits* are encountered most frequently both in Europe

(EFSA, 2007) and in United States (http://www.cdc.gov/mmwr/preview/mmwrhtml/ss5510a1.htm). It is also known as nontyphoidal salmonellosis and typically presents as self-limiting gastroenteritis, although in immunocompromised individuals, serious complications can occur (Boyle et al., 2007).

The infection is localized to the ileum, colon and mesenteric lymph nodes and commonly manifests within 12–72 h after ingestion of contaminated food with diarrhea, vomiting and abdominal pain. Rectal biopsies reveal an acute enteritis characterized by mucosal edema and acute inflammation with polymorphonuclear leukocytes (MacGovern et al., 1979). Bacteremia is uncommon and transient in this syndrome (Mandal et al., 1988).

Bacteremia

Bacteremia (septicemia) is the least common clinical syndrome in man. It is caused by the porcine-adapted *S.enterica* serotype *Choleraesuis* and the bovine-adapted *S.enterica* serotype *Dublin* which may enter the food chain through undercooked pork products or unpasteurized milk, respectively. The syndrome clinically differs from

enteritis in that bacteria are frequently isolated from blood while diarrhea is observed in only about one third of the patients infected with serotypes Dublin or Choleraesuis. Bacteremia is often accompanied by a high spiking fever that distinguishes the syndrome from typhoid fever in which a more continuous fever is observed. Furthermore, bacteremia caused by serotypes Dublin or Choleraesuis often occurs without local manifestations (enteric pathology is encountered rarely), a clinical feature that distinguishes this syndrome from both, typhoid fever and enteritis. Finally, among patients recovering from infection with serotype Choleraesuis, healthy carriers are encountered less frequently than typically described for serotypes causing enteritis or typhoid fever. Thus a rapid clearance of the organism from intestinal sites distinguishes the bacteremic syndrome from both enteritis and typhoid fever (D'Aoust, 2000).

DYNAMICS OF INVASION

Invasive *Salmonella* infections can be caused by many serotypes and begin with a series of events characterized by adhesion and epithelial entry of the *Salmonella* organism.

Adhesion is a process involving the virulence factors known as fimbrae or pili. Invasion of intestinal cells is a hallmark of *Salmonella* pathogenesis and a trait shared by many

serotypes. *Salmonella* is capable of invading non-phagocytic cells of the intestinal epithelium and causing gastroenteritis by multiplying within the gutassociated lymphoid tissue (Peyer's patches). Progression of the illness to a disseminated infection resulting in development of a typhoid-like disease, requires additional access to local mesenteric lymph nodes followed by phagocytic cell uptake for transport to the liver and spleen.

The initial step of the molecular pathogenesis of *Salmonella* consists in enter in the host cytosol and involves modification of the actin cytoskeleton caused directly by *Salmonella* proteins that provoke damage to the membrane and uptake of the bacteria into the host cell. Bacterial products are also able to activate pathways allowing the pathogens to escape from the host defense system (Yan et al., 2003).

ANTIBIOTIC RESISTANCE

Treatment with appropriate antimicrobial agents is crucial for severe cases of salmonellosis or in patients at increased risk of invasive infection. Such patients may include neonates, elderly, transplant recipients, and more in general, immunodeficient (Yan et al., 2003).

The increasing occurrence of antibiotic-resistant *Salmonella* has compromised the therapeutic efficacy of first-line antimicrobial drugs such as ampicillin, chloramphenicol, and thrimethoprim, sulphamethoxazole for the treatment of typhoid and paratyphoid fever.

The high resistance of *S.typhimurium* to ampicillin, chloramphenicol and sulphamethoxazole contrasts sharply with the generally lower level of resistance encountered with other serovars. The acquisition of such resistance probably resulted from the continued prominence of *S.typhimurium* in human disease and the widespread use of ampicillin, chloramphenicol, and sulphamethoxazole in the treatment of bacterial infections. The current situation has developed partly from the use and abuse of antibiotics in human medicine and from the continuing widespread use of medicinally

important antibacterials as growth-promoting or prophylactic agents in the agriculture and aquaculture (D'Aoust, 2000).

MDR *Salmonella* isolates have been reported since the 1960s (Anderson and Lewis, 1965), with the resistance patterns of *Salmonella* serovars of public health importance often associated with specific phage types.

One notable MDR strain is *Salmonella enterica* subsp. *enterica* serovar Typhimurium definitive type 104 (DT104). It was first recognized in the United Kingdom (Threlfall et al., 1994a) and since has been reported in many parts of the world (Besser et al., 1997; Glynn et al., 1998) and from various host species including food animals and pets (Davies et al., 1996; Low et al., 1996) as well as from processed ready-to-eat meat products (White et al., 2001). DT104 strains are commonly known to be pentaresistant, exhibiting resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (Gebreyes et al., 2002).

Mechanism of resistance

While *Salmonella* are intrinsically resistant to some drugs, such as vancomycin (vancomycin molecules are too large to pass through the Gram-negative cell wall),

many resistance mechanisms are acquired through mutation or acquisition of antimicrobial resistance genes. Generally, resistance mechanisms employed by other bacteria toward antimicrobial agents are also applicable to *Salmonella* including production of drug-inactivating enzymes, reduced permeability via the bacterial cell membrane including active efflux pumps, and modification of the cellular target for drug (Yan et al., 2003).

Resistance transferability

The public health concern for the growing prominence of resistance *Salmonella* is heightened by the possibility to transfer such capacity.

Resistance genes can be obtained by bacteria in a number of ways including conjugation, transformation, and transduction (Sefton et al., 2002).

Linked resistance whereby a single resistance (R) plasmid encodes resistance to multiple antibiotics is a problem in both human and veterinary medicine.

An important vehicle by which *Salmonella* and other bacteria acquire antimicrobial resistance genes is via an integron. Integrons are mobile genetic elements that have been found in plasmids, transposons, and integrated into the bacterial chromosome (Bennet,

1999). Integrons consist of multiple genes including: (a) an integrase gene (*intI*), which allows the integron to insert and excise from target DNA (plasmid, transposon, chromosome); (b) promoters for the expression of resistance genes, with an insertion site (*attI*) for antimicrobial resistance gene cassettes to be incorporated into the integron; and (c) genes that code for sulfonamide (SulI) resistance, detergent resistance (QacEΔ1), and an open reading frame with unknown function (Orf5) (Fluit and Schmitz, 1999). Resistance gene cassettes that consist of a resistant gene and a recombination site known as a 59 base element are inserted at the *attI* site (Recchia and Hall, 1997). Transfer of these integron-associated resistance genes occurs through site-specific recombination and has been shown to cross species barriers. This interspecies spread of integron-mediated antimicrobial resistance can be of great concern because resistance genes derived from an increasing number of sources can be transferred to *Salmonella* strains (Yan et al., 2003).

EPIDEMIOLOGY OF *SALMONELLA*

The epidemiology of foodborne diseases has changed in the last two decades because new pathogens has emerged and previously recognized pathogens have increased in occurrence or have become associated with food or new food vehicles.

Between the newly emerged pathogens many are ,or have the potential to be, important foodborne pathogens, including *Escherichia coli* O157:H7 and other enterohaemorrhagic *E. coli*, *S. typhimurium* DT 104, *Cryptosporidium parvum, Cyclospora cayetanensis, Arcobacter butzleri* and *Helicobacter pylor* (Meng and Doyle, 1998).

The ubiquity of *Salmonella* in the natural environment contributes significantly to the continued presence of salmonellae in meat animals and derived products.

Many biological entities, living and dead, act as reservoirs of *Salmonella*, and a diversity of foods have been implicated in outbreaks of food-borne disease. As *Salmonella* lives in the gastro-intestinal tracts of humans and other animals, including birds, it is not surprising that the major food vehicles of transmission are animal-derived

food such as beef, poultry, milk, or eggs, contaminated with animal faeces but any food, including vegetables, may become contaminated (Cox, 2000).

Salmonella may also be found in the faeces of some pets, especially those with diarrhoea, and it is possible to contract infection if fundamental hygienic habits are not applied after contact with pets or pet faeces. Reptiles, such as turtles, lizards, and snakes, are particularly likely to harbour *Salmonella* (http://www.cdc.gov/nczved/dfbmd/disease_listing/salmonellosis_gi.html#6).

Thorough cooking is a way to eliminate *Salmonella*. However, the growing popularity of raw meat dishes such as steak tartare, sushi, and the traditional light cooking of fish and shellfish, indicate that consumer education on foodborne health risks remains a formidable challenge. Although raw pork generally is well cooked to eliminate the threat of *Trichinella* parasites, neglect in the proper handling and cooking if this product can lead to human salmonellosis. The use of raw or undercooked eggs in bakery products frequently is associated with *Salmonella* infections. Raw milk remains an important vehicle of human *Salmonella* and other bacterial infections.

Properly controlled pasteurization eliminates vegetative bacterial pathogens from raw milk.

Fresh fruits and vegetables have gained notoriety in recent years as vehicles of human salmonellosis. The use of fresh or inadequately composted animal wastes as fertilizer, contaminated waters and effluents for field irrigation, and unpotable water for post-harvest washing and production of ice for cold shipment of perishable products, likely contributed to outbreaks attributed to fresh products.

Salmonella may survive to antibacterial treatments applied to seeds and can proliferate during seed germination (D'Aoust, 2000).

INCIDENCE OF *SALMONELLA* IN FOOD

"The Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents, Antimicrobial Resistance and Foodborne Outbreaks in the European Union in 2006", published by the EFSA in 2007 reported the data on the occurrence of *Salmonella* in various foodstuff.

FOOD CATEGORY	n. of samples	% pos
Fresh broiler meat	33,257	5.6
Fresh broiler meat in countries with a monitoring/surveillance programme	20,567	4.1
Broiler meat preparation and product		
• Non-ready-to-eat	6,760	6.7
• Ready-to-eat	6,191	0.4
Fresh turkey meat	7,141	6.4
Table egg	28,773	0.8
Fresh pig meat	58,330	1.0
Fresh pig meat in countries with a monitoring/surveillance programme	46,018	1.3
Pig minced meat preparation and product		
• Non-ready-to-eat	28,973	1.4
• Ready-to-eat	22,491	0.5
Fresh bovine meat	53,450	0.2
Fresh bovine meat in countries with a monitoring/surveillance programme	25,419	0.1
Bovine minced meat, meat preparation and product samples		
• Non-ready-to-eat	20,425	0.5
• Ready-to-eat	4,750	0.4
Cheeses	12,252	0.05
Vegetables, fruits and herbs	7571	0.14

Source EFSA 2007

The majority of the reported food-borne *Salmonella* outbreaks were related to eggs while meat (poultry and pig) was the second most common cause. In animal populations, *Salmonella* was most frequently detected in poultry flocks.

Most of the countries providing data on *Salmonella* in fresh broiler meat in 2006, reported substantial numbers of positive samples. The proportions of positive samples varied between different stages of the production line: at slaughter ranged from 0% to 15.1%; at processing plants, from 0 to 13.3%; at retail level, between 2.5% and 10.3%.

As regards to *Salmonella* in eggs and eggs products, in general *Salmonella* was found in fresh eggs, raw material at processing and at retail. The findings based on single sampling testing ranged from 0% to 7.1%.

The results of investigations of fresh pig meat carried out in 2006 indicate that *Salmonella* was found in moderate proportions of pig meat, up to 13%. Data reported by countries that apply control programmes have showed very low levels of *Salmonella* contamination in fresh pig meat.

As regards to the *Salmonella* occurrence in non ready-to-eat products of pig meat origin, the overall prevalence was included between 0 and 4.8%, and the highest proportion of positive samples was reported by Italy in minced meat. As regards to ready-to-eat meat,

meat preparation and product samples the overall prevalence was included between 0 and 3.4%, with the highest proportion reported from Italy.

In bovine meat the proportion of positive samples was very low (< 1.0%) in most reporting countries. However, from Spain several studies were reported showing proportions from 0.7% to 7.5% with the highest proportions among samples originating from slaughterhouses.

In compound feedingstuffs (final products), including both domestic and imported products, the proportion of *Salmonella* positive findings ranged from none to 9.4% in cattle feed, 3.3% in pig feed and 5.3% in poultry feed.

The level of *Salmonella* contamination in feed material of vegetable origin indicated that 2.5% of the samples/batches of oil seeds and products thereof were positive in 2006 and since 2002 this feed material has been consistently the most contaminated one.

This finding generally indicates that oil seeds like soybean, rape, sunflower and products thereof probably are the most likely sources of *Salmonella* in animal feed.

INCIDENCE OF SALMONELLOSIS IN HUMANS

According to the EFSA report (2007), *Salmonella* resulted to be the second cause of foodborne gastroenteritis in Europe, after *Campylobacter spp.*. In 2006, a total of 165,023 confirmed cases of human salmonellosis were reported via TESSy (The European Surveillance System) from 31 countries: 25 EU MS and six non-MS. The number of human salmonellosis cases in the EU reported via BSN (Basic Surveillance Network) has decreased since 2004; from 196,042 (42.2/100,000) in 2004 to 173,879 confirmed cases (38.2/100,000) in 2005 and to 160,649 (34.6/100,000) in 2006. This represents a 7.6% decrease from 2005 and 18.1% decrease from 2004 in EU MS.

Reported salmonellosis cases in humans indicating: total number/confirmed cases/incidence reported to BSN in 2006/total number reported through Enter-net in 2006

COUNTRY	Cases	Confirmed cases	Cases/100,000	Enter-net
Germany	52,575	52,575	63.8	2,703
Czech Republic	25,102	24,186	235.9	24,521
United Kingdom	14,055	14,055	23.3	14,468
Poland	13,362	12,502	32.8	-
Hungary	9,752	9,389	93.2	6,240
Slovakia	8,784	8,242	152.9	8,990
France	6,339	6,339	10.1	5,897
Italy	5,164	5,164	8.8	3,412
Spain	5,117	5,117	11.7	4,659
Austria	4,787	4,787	57.9	5,300
Sweden	4,056	4,056	44.8	4,075
Belgium	3,630	0	0	3,630
Lithuania	3,557	3,479	102.2	3,597
Finland	2,574	2,574	49. 0	2,578
others	8,386	8,184	27.4	7,12
Tot Europe	167,240	160,649	34.6	97,190

In spite of this reduction, *Salmonella* is still a serious problem for public health, most of all considering that the data about food-borne infections recorded by the various national surveillance systems, is underestimated because of the problem of the under notification.

The risk for infection is highest in the age group four years of age and younger and is almost three times that of the next highest risk age group (ages five to 14) and five to seven times higher than for those aged 15 and older.

Salmonella infection can cause epidemic outbreaks in places crowded with people (e.g. schools, hospitals, rest homes), or when different people can use the same food sources (e.g. restaurants, refectories).

Salmonella episodes can take place in small outbreaks, but more often isolated cases are detected (http://www.epicentro.iss.it/problemi/salmonella/epid.asp).

A peak in the number of reported cases occurs in the summer and autumn, with a rapid decline into the winter months. This pattern supports the influences of temperature and behaviour (i.e. food consumption habits such as barbequing) on S*almonella* incidence

True estimates of numbers affected are fraught with difficulty, but the most recent data about typhoid disease according to the WHO indicate that there are over 20 million cases and 200.000 deaths worldwide each year (Crump et al., 2004). The death rate for persons with multidrug-resistant infections was estimated to be 10 times higher in the two years following specimen collection than for the general population (http://www.who.int/mediacentre/factsheets/fs139/en/).

In 2006, as in the previous years, the most common serotypes were *S. enteritidis* e *S. typhimurium*. The most frequent phage type of *S. enteritidis* in 2006 was PT4, while of the *S. typhimurium* phage types, DT104 remained the most prevalent (EFSA, 2006).

SALMONELLA IN THE PIG PRODUCTION CHAIN

Over the last decade, pork has gained recognition as a source of human salmonellosis (Lo Fo Wong et al., 2002).

S. choleraesuis was the first *Salmonella* serotype isolated from pigs (Salmon and Smith, 1886), only 2 years after the first isolation ever of *Salmonella*, performed by Gaffky in 1884 (Le Minor, 1994). In the course of time, more than 2400 different serotypes were isolated from different animal species, including pigs.

The pathogenesis in pigs of infection with broadhost range serotypes of *Salmonella* was largely neglected until recently. As previously said, infection of pigs with the swine adapted serotypes Typhisuis and Choleraesuis usually result in swine typhoid, characterized by severe systemic disease that is often fatal, but infected pigs generally carry *Salmonella* serotypes asymptomatically in the tonsils, the intestines and the gut-associated lymphoid tissue (GALT) being a major reservoir of *Salmonella* and posing an important threat to animal and human health (Boyen et al., 2008).

PATHOGENESIS OF NON-TYPHOIDAL *SALMONELLA* IN PIGS

Transmission of *Salmonella* between pigs is thought to occur mainly via the faecal–oral route. Some studies showed that the upper respiratory tract and lungs may be a portal of entry as well (Boyen et al., 2008). However, a study by Fedorka-Cray et al. (1995) demonstrated that *Salmonella* can enter the host via the tonsils, the nasal-associated lymphoid tissue (NALT), and lungs, bypassing traditional oral-fecal dissemination.

- COLONIZATION OF THE UPPER GASTROINTESTINAL TRACT

Porcine epithelial beta-defensin 1 is expressed in the dorsal tongue at antimicrobial concentrations and may contribute to the antimicrobial barrier properties of the dorsal tongue and oral epithelium. Salmonellae that overcome this barrier may colonize the tonsils. The palatine tonsils are often heavily infected in pigs and should, therefore, not be underestimated as a source of *Salmonella* contamination during slaughter (Boyen et al., 2008). In several studies (Davies et al., 1999; Swanenburg et al., 2001c; Bonardi et al., 2003; Vieira-Pinto et al., 2005) an occurrence between 8 and 36.4% was found in the tonsils. During ingestion, *Salmonella* enters the tonsils in the soft palate and persists in the tonsillar crypts. Surprisingly, no detailed information has been gathered on how

Salmonella interacts with and persists in the porcine tonsillar tissue, although some observations mention persistence of *Salmonella* on the superficial epithelium of the tonsillar crypts (Horter et al., 2003).

Following ingestion, *Salmonella* must survive the low pH of the stomach. It has been shown that salmonellae can adapt to and survive in acidic environments up to pH 3 by producing acid shock proteins (Audia et al., 2001; Smith, 2003; Berk et al., 2005). The non-glandular region and the cardiac gland zone of the porcine stomach have a pH range between 5 and 7 (Boyen et al., 2008). Nevertheless, since the pH of the fundus and pylorus of the porcine stomach in normal conditions decreases to pH 2 or even lower, many bacteria will be killed. When the pigs are fed a coarsely ground meal, this will result in a slow emptying of the stomach and consequently a longer stay in the acidic environment, reducing the number of surviving bacteria. In addition, it has been determined that the lethal effects of the porcine stomach contents are pH-dependent but that low pH is not the sole killing mechanism (Boyen et al., 2008).

- COLONIZATION OF THE LOWER GASTROINTESTINAL TRACT

Bacteria that survive passage through the stomach, travel to the small intestine where they encounter other antibacterial factors including bile salts, lysozyme and defensins.

Even though *S.typhimurium* can be highly resistant against the direct antibacterial effects of bile salts, these salts repress the invasion of *Salmonella* in epithelial cells, possibly by decreasing virulence gene expression (Prouty and Gunn, 2000). Since high concentrations of bile salts are present in the upper part of the small intestine, this might explain why *Salmonella* preferentially colonizes the ileum, caecum and colon (Boyen et al., 2008). In the distal parts of the intestine, adherence to the intestinal mucosa is generally accepted as the first step in the pathogenesis of *Salmonella* infections in pigs. Following adhesion, *Salmonella* invades the intestinal epithelium. It has been shown that *Salmonella* can invade porcine absorptive enterocytes, M-cells and even goblet cells. *S. typhimurium* is found within the porcine enterocytes and mesenteric lymph nodes at 2 h after oral inoculation (Reed et al., 1986; Fedorka-Cray et al., 1995). Recently, it has been shown that the virulence genes encoded in the *Salmonella* Pathogenicity Island 1 (SPI-1) mediate this invasion step and that these genes are crucial for the colonization of the gut and GALT (Boyen et al., 2006; Brumme et al., 2007). The rapid growth of *S.typhimurium* in the porcine gut and subsequent induction of proinflammatory responses may explain why pigs in most cases confine *S.typhimurium* infection to the intestines, whereas slow replication of *S.choleraesuis*

may enable it to evade host immunity and subsequently spread beyond the intestinal boundaries (Paulin et al., 2007).

- THE MECHANISM OF *SALMONELLA*-INDUCED-DIARROHEA

When salmonellae invade the intestinal epithelium, the production of several cytokines, is induced in the porcine gut (Uthe et al., 2007; Volf et al., 2007). IL-8 is the most extensively studied, and for *S. typhimurium*-induced diarrhoea, probably the most important of these cytokines. Infection of porcine intestinal epithelial cells and porcine macrophages (Volf et al., 2007) with *S. typhimurium* increases IL-8 secretion by these cells. IL-8 plays an important role in the initial movement of neutrophils (PMN) from the circulation into the subepithelial region (McCormick et al., 1995).

The tools available for *Salmonella* to induce diarrhoea are overwhelming. Keeping this in mind, it may seem rather peculiar that most of the *S. typhimurium* infections in pigs are subclinical and asymptomatic. Apart from factors such as the infection pressure, the age and immunological status of the host, again *Salmonella* SPI-1 effectors may play a role (Boyen et al., 2008).

- SYSTEMIC SPREAD

The systemic part of a *S. typhimurium* infection in pigs is not well-documented. It is generally accepted that *Salmonella* can spread throughout an organism using the blood stream or the lymphatic fluids and infect internal organs, although this has not yet been studied in swine. The colonization of the mesenteric lymph nodes, spleen and liver can result in prominent systemic and local immune responses (Dlabac et al., 1997). Macrophages are the cells of interest for host-restricted or –adapted *Salmonella* serotypes to disseminate to internal organs. The bacteria replicate rapidly intracellularly and cause the systemic phase of the infection, while interfering with the antibacterial mechanisms of the macrophages and inducing cell death (Waterman and Holden, 2003; Hueffer and Galan, 2004).

- PERSISTENT *S.TYPHIMURIUM* INFECTIONS IN PIGS

Infections of pigs with *S. typhimurium* may result in long-term asymptomatic carriage of these organisms (Wood et al., 1991). Since this carrier state in pigs is difficult to detect in live animals, either by bacteriological or serological methods (Baggesen and Wegener, 1993; Nollet et al., 2005), these pigs can bias monitoring programmes. Stress-

induced excretion of *S. typhimurium* by carrier pigs transported to the slaughterhouse may cause contamination of shipping equipment and holding areas, resulting in preslaughter transmission of *Salmonella* to non-infected pigs (Berends et al., 1996; Lo Fo Wong et al., 2002; Boughton et al., 2007). Although the mechanism of this stress-induced excretion is not known, there are some indications that catecholamines may play a role. It has been shown that *S. typhimurium* can ‘‘sense’’ catecholamines and as a result increase its growth rate (Rahman et al., 2000; Williams et al., 2006).

Very few researchers have made an attempt to unravel the mechanism of the concealed, but prolonged infection in carrier pigs (Boyen et al., 2006; Wang et al., 2007).

Research in the mouse model suggests that *Salmonella* may reduce its own intracellular growth rate and may actively limit its impact on the infected tissues as if it was a commensal. It was found recently that *S. typhimurium* evades strong host responses by downregulating the local inflammatory response in pigs (Boyen et al., 2008; Wang et al., 2007). Also in pigs, it has been suggested that subversion of the dendritic cell function by *S. typhimurium* can prevent efficient stimulation of T-cell proliferation (Wang et al., 2007).

SALMONELLA SURVEILLANCE PROGRAMMES IN PIG AND PORK

An increase in human outbreaks of salmonellosis, originating from infections in animals, has been seen periodically.

Pork is one of the main sources for human salmonellosis (Wegener and Baggesen, 1996; Berends *et al.,* 1998b; Fedorka-Cray *et al.,* 2000), being the source of approximately 20% (5-30%) of the human cases (Steinbach and Hartung, 1999). Therefore, reduction of *Salmonella* risks associated with pork can significantly contribute to the protection of human health.

Attention has been increasingly focused on the prevention and control of *Salmonella* in animal production, by different organizations such as World Health Organization (WHO, 1993), World Organization for Animal Health (Office International des Epizooties – OIE) and the EU (Dir 92/117/EEC). The need for global cooperation in the control of salmonellosis was also emphasized (Bögel, 1991).

Globally, the basis of modern longitudinal and integrated food safety assurance (LISA) is a novel approach designed to prevent potential food safety problems before they actually appear, by intervening at points of the food chain where they are expected to appear (Mossel, 1999). As said, each link in the food chain has its share of

responsibility for reducing the risk of salmonellosis. Obviously, hazards have to be controlled at relevant, multiple points in a coordinated way. Where they cannot be totally eliminated, public health risks can be reduced and it is possible to achieve a "summation effect" of risk reductions in such a longitudinal and integrated system that results in an ultimate risk reduction.

In 1980, WHO formulated three lines of defence for the control of *Salmonella* which are still valid (WHO, 1980):

1. control of *Salmonella* in the food producing animal: pre-harvest control;
2. improvement of hygiene during slaughter and further processing of meat: harvest control;
3. final preparation of the food by education of the industry and consumer to obtain application of correct hygienic principles at consumer level: post-harvest control.

In the EFSA report about "Risk assessment and mitigation options of *Salmonella* in pig production" (2006), the definition of harvest covers the part of the food chain beginning with the transport of the slaughter animals from the farm gate, the lairage phase, slaughtering itself, up to the cooling of the carcasses. The farmer usually can influence

the status of the slaughter animals only up to the point of transport to the slaughterhouse. Therefore harvest is separated from pre-harvest at this stage. The post-harvest level includes cutting and processing, production of raw, fermented or "safe products" (in respect to *Salmonella* contamination) up to retail and consumer levels.

A successful prevention of food borne salmonellosis originating from pork has to involve all those three lines.

Today it also seems to be generally accepted that, both from an economic and an epidemiological point of view, it is necessary to focus on the control at the production level. The previously supported strategy that it is possible to control *Salmonella* only at consumer level, have been abandoned (Wierup, 1995).

European law and directives, applicable to every Member State, will ensure a surveillance programme for *Salmonella* in pig husbandry before 2009 (Anonymous 2003a,b).

Regulation (EC) No 2160/2003 on the control of *Salmonella* and other specified zoonotic agents provides for the setting of Community targets and for reducing the prevalence of *Salmonella* serovars with public health significance in pig herds.

According to this Regulation, the targets shall include: 1) the maximum time limits within which the targets shall be reached; 2) the definition of epidemiological units; 3) the definition of the testing schemes necessary to verify the achievement of the targets; 4) where relevant, the definition of the *Salmonella* serovars with public health significance. The Regulation states that before proposing such rules on specific control methods, the Commission shall consult the EFSA.

Within 18 months following the setting of the targets, Member States shall prepare and submit national control programmes and the Commission shall approve them. The timetable for setting targets for pig production is December 2007 for breeding pigs and December 2008 for slaughter pigs (the order may be reversed if this is more appropriate based on the scientific data available). When defining Community targets for pig production, the Commission shall provide an analysis of the expected costs and benefits, taking into account in particular certain criteria laid down in the Regulation.

Before proposing targets, comparable data on *Salmonella* prevalence within the Community shall be available. It may be decided to establish rules concerning the use of specific control methods, such as vaccines, anti-microbials or substances influencing the porcine intestinal flora environment, in the context of the control programmes.

The Community legislation allows establishment of control measures at stages of the food-chain after primary production.

RISK FACTORS ALONG THE PIG PRODUCTION CHAIN

In order to be able to design an adequate intervention strategy for *Salmonella* infections in pigs, risk factors for the occurrence of this infections need to be investigated.

A lot of research has focused on risk factors associated with *Salmonella* prevalence in pig herds, based mainly on bacteriological testing (van der Wolf et al., 2001b), with the general aim to identify possible control measures to reduce the *Salmonella* prevalence in slaughter pigs and thereby to guarantee safe pork (Nollet et al., 2004).

- ***PRE-HARVEST LEVEL***

The main factors influencing *Salmonella* contamination of finishing pig reported in the literature are (Balœil et al., 2004):

1) Level of hygiene:
   - washing hands;
   - controlling for birds, flies and rodents;
   - handling of manure.
2) Herd management:
   - size of the herd;

- batch production system;
- housing (type of pen partitions and wall separation).

3) Feeding practices:

- groundness and pH of feed;
- type of feeding (wet versus dry).

4) Health disorders:

- parasite infestation;
- use of antibiotics;
- health status of the herd.

Salmonella infections can be managed by strict application of all-in/all-out procedures including cleaning and disinfections (Balœil et al., 2004).

At the herd level, controlling birds, flies and rodents in the stables and storage facilities as well as keeping pets such as cats and dogs out of the pig house, will help to prevent the introduction of *Salmonella* from the environment (Lo Fo Wong et al., 2002).

The herd is a complex factor that also includes all related factors, such as the frequency of introduction of animals, the number of supplier herds, the size of the production area, the number of units, and a larger supply of feed and water.

Only few studies have confirmed that an increased herd size imposes an increased risk of *Salmonella* infection (Dahl, 1997; Carstensen and Christensen, 1998). However, an increase in herd size does not necessarily mean an increase in pig density at the pen level. In most countries, pen density is restricted by legislation and larger herds basically are comprised of more epidemiological units. In fact, larger operations might have the resources necessary for the implementation of effective biosecurity measures and good manufacturing practice (Lo Fo Wong et al., 2004). This was supported by a study conducted by van der Wolf et al. (2001b), who found an increased risk for *Salmonella* in small-to-moderate-size herds (<800 finishers) compared to large herds.

Herds are subject to the introduction of feed and new stock, and as such are exposed to potential sources of infection. In a risk factor study carried out by Lo Fo Wong et al. (2001), it was found that the more supplier herds supplying animals to a finishing herd, the larger the probability of testing animals seropositive in the receiving herd.

To guard against the co-introduction of *Salmonella* through purchased animals, *Salmonella*-free breeding flocks should be identified or established through a certification system or through weaning in a clean environment. In addition, the number of supplier herds should be kept to a minimum. Batch production with efficient cleaning

and disinfection procedures between batches in combination with the use of a hygienic lock, i.e. sanitary facilities for washing hands and changing clothes and boots, should be made standard operating procedures for all (indoor) pig production to avoid the introduction and/or spread of *Salmonella*, as well as other pathogens, in pig herds (Lo Fo Wong et al., 2002).

The type of floor, was examined by some authors (Davies et al., 1997; Nollet et al., 2004): a fully slatted floor resulted to be protective because the faeces from pigs housed on this type of floor immediately flows away in the manure pit, so pigs cannot have a lot of contact with faeces.

As said, faecal-oral transmission is the most-likely mode of transmission of virulent salmonellae (Boyen et al., 2008). Dahl et al. (1996) showed that closed pen separations posed a barrier which prevented faecal contact between adjacent pens and thereby spread of infection. It was demonstrated that snout contact is significantly associated with seropositivity (Lo Fo Wong et al., 2002 and 2004).

As regards to feeding management, the main variable taken into account are:

- type of feed;

- feeding meal.

The protective effect on *Salmonella* seroprevalence of non-pelleted feed compared to pelleted feed was reported in several studies (Dahl, 1997; Harris et al., 1997; Lo Fo Wong et al., 2002 and 2004; Hautekiet et al., 2008): coarsely ground grain digests slower than more finely ground pelleted feed. As a result, some parts of the carbohydrates of non pelleted-feed will be fermented in the large intestine, forming volatile fatty acids. These provides a hostile microbiological ecosystem for *Salmonella*.

The beneficial effect of wet feed was demonstrated by several studies (Van der Wolf et al., 2001b; Balœil et al., 2004; Hautekiet et al., 2008), and is probably due to the production of lactic-acid producing bacteria as a result of the natural fermentation process. These acids lower the pH of the feed to a level at which *Salmonella* bacteria do not multiply.

Various concurrent infections might favour *Salmonella* infection: respiratory viruses, such as porcine reproductive and respiratory syndrome virus (PRRSV), could induce immunodepression which could facilitate *Salmonella* contamination and multiplication (Balœil et al., 2004). A synergism between PRRS virus and S. choleraesuis was

observed experimentally by Wills et al. (2000). Enteric health problems caused by pathogens (e.g. Lawsonia intracellularis) might predispose to subclinical salmonellosis due to a disturbance of the ecological balance of the gut flora (Balœil et al., 2004).

- ***HARVEST LEVEL***

TRANSPORT AND LAIRAGE

In several trials, between 0-20% of *Salmonella*-free pigs became infected during transport to the slaughterhouse and subsequent lairage. Furthermore, the studies also showed that within 2-6 hours of transport and lairage the number of animals that excrete *Salmonella* can sometimes more than double (1.0-2.4 times). This group will consist of pigs with "new" infections, pigs with reactivated latent infections (*Salmonella* still present in the lymph nodes), and pigs that were already excreting the organism at the farm. The physiological reactions to the several stress factors imposed by transport (noise, smells, mixing with unfamiliar pigs, high stocking densities, long duration of transport etc.) can have a strong influence on the *Salmonella*-status of the animals. Stress induces *Salmonella* carriers to start shedding again and also induces *Salmonella*-free pigs to be more susceptible to stress (Berends et al., 1996; Zengh et al., 2007); as a

consequence, their immune status can be impaired and the number of pigs excreting *Salmonella* can significantly increase upon arrival at the abattoir (Berends et al., 1996; Lo Fo Wong et al., 2002).

Many of the same stress factors present during transport are also in force during the waiting time in lairage, and the proportion of pigs shedding *Salmonella* has been shown to increase with the length of time spent in lairage (Berends et al., 1996; Lo Fo Wong et al., 2002). Fedorka-Cray et al. (1995) showed that *Salmonella* can be isolated from mesenterial lymph nodes and caecal and rectal contents already 3 hours after infection, which makes it possible for pigs to start excreting *Salmonella* before they are slaughtered.

Cleaning and disinfection of trucks and lairage pens can only prevent cross contamination with *Salmonella* from other farms, but not with those already present in the group (Berends et al., 1996; Nowak et al., 2007; Magistrali et al., 2008).

SLAUGHTER

Salmonella prevalence in slaughter pigs has been investigated in a number of studies (Swanenburg et al., 2001a and c; Bonardi et al., 2003; Botteldoorn et al., 2003; Vieira-Pinto et al., 2005; Magistrali et al., 2008).

If the slaughter process is carried out perfectly, pigs with *Salmonella* in their intestines will theoretically not contain *Salmonella* on their carcasses or in the meat after slaughter. Slaughter hygiene and slaughtering according to HACCP/GMP principles are important to prevent cross contamination of carcasses during the slaughter process. Critical control points in the slaughter line must be known before implementing additional hygienic measures in the slaughter process (Swanenburg et al., 2001a).

Depending on the many influential factors described previously, the *Salmonella* status of the pigs entering the slaughter line may vary considerably between days or even batches.

In a study conducted by Berends et al. (1997) the risk factors that lead to the contamination of carcasses with *Salmonella* in the pig slaughterline were analyzed and quantified.

The pig as a "risk factor".

The faeces of recently infected pigs may contain several thousands to several millions cfu *Salmonella*/gram, since are particularly important in relation to carcass contamination. Within groups of pigs slaughtered there is a strong correlation between the proportion of animals with *Salmonella* in their faeces and the proportion of contaminated carcasses at the end of the line. This has been demonstrated even more recently (Bonardi et al., 2003; Botteldoorn et al., 2003; Vieira-Pinto et al., 2005).

The attributable fraction of pigs with *Salmonella* in their faeces has been estimated at approximately 70%. In other words, about 70% of all carcass contamination results from pigs themselves being carriers and about 30% because other pigs in the line were carriers (cross contamination) (Berends et al., 1997).

The process as a "risk factor".

In most slaughterhouses pig carcasses are dressed with the skin still on. This requires some initial processing of the carcass with the primary purpose of removing the hair. First comes the scalding process, followed by dehairing, singeing and, finally, polishing (Lo Fo Wong et al., 2002).

Scalding and singeing result in virtually complete elimination of the skin contamination that existed before slaughter.

Ordinarily, the number of *Salmonella* is reduced during scalding (Gill and Bryant, 1992, 1993). However, if the water temperature drops below the recommended 62°C and/or the amount of organic material is sufficient to protect the bacteria against the heat, the risk of bacteria surviving this process is increased, transforming the scalding process into a critical site of contamination (Swanenburg et al., 2001a). The dehairing process is commonly regarded as a site for recontamination of the scalded carcasses (Gill and Bryant, 1992, 1993; Borch et al., 1996). The rotating flails that are removing the hair may squeeze faeces from the anus, potentially contaminating the equipment with faecal micro-organisms, including *Salmonella* (Borch et al., 1996). Berends et al. (1997) showed that scalding and dehairing usually reduce the number of carcasses with *Salmonella* on the skin with 50% (10-70%).

Singeing at 1300–1500 °C reduces surface contamination of the carcass. However, bacteria in certain areas, such as in the deeper skin, the base or orifices of the ears or in the hair follicles, may survive (Berends et al., 1997; Gill, 1998). These bacteria can then

be redistributed over the entire carcass during polishing by the rotating flails and brushes of the polisher (Lo Fo Wong et al., 2002).

In the dressing process, two processes in particular have been identified as critical control points: the evisceration process, including bung dropping, and the removal of the pluck set (tongue, oesophagus, larynx, trachea, lungs, heart and liver). The carcass splitter is not normally considered to be an important source of carcass contamination (Berends et al., 1997; Gill and Jones, 1997). However, it may become persistently contaminated with *Salmonella* and as such will be a source for carcasses contamination (Sørensen et al., 1999; Hald et al., 2001). In fact, contaminated slaughterhouse equipment seems to play a more important role in the final carcass contamination level than the slaughterhouse personnel (Hald et al., 2001), partly due to the possible build up of bacteria in or on the equipment during working hours (Yu et al., 1999; Hald et al., 2001), especially during warm summer months (Hald et al., 2001).

Current evisceration practices contribute to an estimated 55-90 % of total carcass contamination with *Salmonella*, while the polishing equipment contributes to an estimated 5-15 %.

In conclusion, it can be inferred that splitting, dressing and meat inspection together may contribute between 5-30%.

After a pig has entered the slaughter process, the final *Salmonella* contamination of the dressed carcass originates from the combined impact of several sources which can be summarized as: 1) the animal itself; 2) previously slaughtered animals via the processing machinery; 3) slaughterhouse personnel; 4) persistently contaminated equipment (Lo Fo Wong et al., 2002).

- ***POST HARVEST LEVEL***

Although manufacturing and retail levels of pork production depend very much on the quality of the raw materials and products that are received, proper and sensible handling of raw materials during is vital to successfully avoid cross-contamination between products. In addition, time and temperature abuses may create situations that support the survival and propagation of micro-organisms that may be present in foods (Lo Fo Wong, 2002).

RISK-BASED SURVEILLANCE: THE DANISH EXPERIENCE

Various *Salmonella* surveillance programmes has been started in European countries.

In Denmark a control programme was initiated in 1993 by the Danish Ministry of Food, Agriculture and Fisheries in response to increasing numbers of human cases of salmonellosis attributable to pork consumption (Alban et al., 2002; Mousing et al., 1997). This on-going programme is based on the serological surveillance of all herds that produce more than 200 pigs per annum and their subsequent assignment into one of three levels of a Serological *Salmonella* Index (SSI). SSI levels one to three represent low, medium and high levels of *Salmonella* in the herds, respectively, with level two and three herds paying penalties and undergoing on-farm investigations. The programme's objective is to lower the prevalence of *Salmonella* so that domestically produced pork is no longer an important source of salmonellosis in humans (Mousing et al., 1997). Since 2001 the prevalence of *Salmonella* in Danish pork (monitored at the slaughterhouse) has reduced from 1.5 to 1% of carcass swab samples taken.

The number of cases of salmonellosis in humans in Denmark attributable to pork consumption decreased from 1,444 in 1993 to 142 in 2004 (Benschop et al., 2008).

Since 2004 the cost-effectiveness of *Salmonella* surveillance has received great attention. A simulation study using data from 2001 to 2002 of the Danish programme found that the number of samples taken from low prevalence herds could be reduced without exposing food safety to risk. This lead to the development of a 'risk-based' approach to *Salmonella* surveillance implemented in mid-2005. Herds with no positive samples from the previous 3 months testing are sampled once per month instead of the previous random sampling based on herd size (Benschop et al., 2008).

Many European studies have investigated risk factors such as herd size, feed type and hygiene, for salmonellosis in pig herds (van der Wolf et al., 2001b; Belœil et al., 2004; Lo Fo Wong et al., 2004; Nollet et al., 2004). The aim of such investigations was to assess the relationships between certain farm characteristics, managerial and hygiene practices, pig-health status and *Salmonella* shedding by pigs at the end of the finishing period (Belœil et al., 2004). The investigations consisted basically in two steps: 1) collection of standardised risk-factor questionnaire including questions on herd size and type, housing conditions, management practice, feeding practice, hygiene practice, cleaning and disinfection procedures, health and disease prevention, and production parameters; 2) collection and analysis of blood samples to determine the subclinical

Salmonella status of finishing-pig herds. Serological results were combined with the questionnaire information. The outcome variable was defined as the number of seropositive samples out of the number of samples taken per herd (i.e. *p*/*n*). Continuous explanatory variables were tested for a linear relationship with the outcome (Lo Fo Wong et al., 2004).

In other studies (Nollet et al., 2004), the herd-level risk factors for the prevalence of *Salmonella* carriers in slaughter pigs was based on the isolation of *Salmonella* in the mesenteric lymph nodes, immediately after evisceration.

EXPERIMENTAL STUDY

THE AIM OF THE STUDY

Salmonella prevalence in pig meats at slaughterhouse can be comprised in a range between <0,1 -32,8% (EFSA, 2006) owing to different factors (Lo Fo Wong et al., 2002).

The main risk for the spread of *Salmonella* is represented by asymptomatic carrier pigs, that cannot be recognised as infected at farm level and, under normal conditions, do not shed *Salmonella* in their faeces. When these animals are strongly stressed by transport or poor handling, their immune status can be impaired and *Salmonella* can be excreted with the faeces, increasing the risk of cross-contamination between pigs during transport and lairage in the abattoir (Nowak et al., 2007). Subsequently, *Salmonella* can be transmitted through inadequate hygienic practices. Furthermore, the capacity of some strains to become resident enable cross-contamination between carcasses and with the slaughterhouse environment (Swanenburg et al., 2001a).

The new European rules concerning the microbial criteria for food safety (Reg. EC 2073/2005) give to the food business operator the responsibility of food safety control. They should ensure food safety by a preventive approach, such as implementation of good hygiene practice and application of procedures based on hazard analysis and critical control point (HACCP) principles. Microbiological parameters can be used in validation and verification of HACCP procedures and other hygiene control measures. As regards to this, microbiological criteria have been established to define the acceptability of the processes, and also the limit above which a foodstuff should be considered unacceptably contaminated with the microorganisms.

At slaughterhouse *Salmonella* is considered a process hygiene criteria and therefore it must be evaluate on pig carcasses at the end of the process, after dressing but before chilling. In case of the *Salmonella* presence above the limits, the food business operator must conduct an analysis to evaluate the source of contamination and implement corrective actions, including the review of process controls and of origin of animals.

In Sardinian slaughterhouses local and from other Italian regions and from EC pigs are slaughtered, but there is a lack of data about *Salmonella* prevalence at farm level and pork meat.

The main objectives of our study were:

1. to evaluate *Salmonella* prevalence in slaughtered pigs and the environment of the abattoir, in relation to the lay-out and the hygienic and handling slaughtering procedures;

2. to determine the sources of direct and cross-contamination by *Salmonella* of swine meat at slaughterhouse;

3. to characterize the *Salmonella* isolates by phenotypic and genotypic techniques in order to determine clonal relationships between the isolates and consequently obtain information about the traceability of the pathogen in the pig chain.

A further part of the study was realized at farm level in order to evaluate the presence of asymptomatic carriers pigs shedding *Salmonella* in their faeces; the same animals were sampled at slaughterhouse too.

EXPERIMENTAL DESIGN

Our survey was carried out from June 2006 to February 2008.

Experiments were conducted in two finishing swine farms (F1 and F2) and in five EC pig slaughterhouses (S1, S2, S3, S4 and S5).

In the first part of the study *Salmonella* prevalence has been evaluated only at slaughterhouse level. Subsequently, considering the results obtained we extended our survey at farm level.

Farms selection

F1 is located in the central part of Sardinia while F2 in the south-west of the island. Farms were strategically chosen because their pigs were slaughtered in two of the abattoirs included in our investigation.

Both farms were intensive, indoors farms; however, some differences could be detected between the two farms. The main difference was the herd size: F1 was a small farm with n.30 swine heads, 5 of which were sows. and 1 boar; F2 was a very large farm,

with n.18,000 swine heads, 1,500 of which were sows and 10 boars Pigs were housed in 30 buildings.

The housing conditions were similar: pigs were housed in group-housing while pregnant sows were confined in sow stalls. In F1 pigs were divided into three units, lodged in the same building. Pens were separated with solid walls about 1.5 m high. In F2 pigs are housed into 5 buildings. In this farm too, pens were separated with solid walls about 1.5 m high. In both farms the floor was of the fully slatted kind and the all in/all out strategy was applied. Pigs were feeded with purchased pelleted feed; there were five different kinds of feed, one for each growing phase, based on the weight of the pig:

- From birth to 6-8 kg (pigs still with the sow);
- Pre-starter: to 8-11 Kg;
- Starter: from 11 to 25 Kg (weaning age);
- From 25 to 35 Kg;
- 35 Kg-slaughtering (circa 110 Kg).

Pigs were slaughtered at the weight of ca. 110 kg.

Slaughterhouses selection

The choice of the slaughterhouses was aimed to cover a quite large part of the region, and high capacity of pig slaughtering plants were included. S1 is located in the north of Sardinia, S2, S3 and S4 in the central part and S5 in the south-west.

The slaughterhouses were comparable from the point of view of the slaughter techniques and procedures (stunning, bleeding, scalding, flaming, polishing, bung removal, evisceration, pluck removal, splitting of carcass, veterinary inspection) and for the number of pigs slaughtered per hour. All the abattoirs slaughtered Sardinian pigs and pigs coming from other parts of Italy or the EU.

Sampling collection

At farm level we sampled:

1. Feed
2. Pig faeces

As previously said, feed are a potential source of introduction of *Salmonella* and play a role in the establishment of infection in the animal (Lo Fo Wong et al., 2002). The

faeces were taken from individual pigs in order to determine the *Salmonella* status of the pigs before slaughtering.

Both farms were visited twice; during each visit samples of feed and faeces were taken. Pigs whose faeces were collected at farm were subsequently sampled at slaughter: pigs from F1 at S4 (during the first sampling day) and S3 (during second sampling day); pigs from F2 at S5 during both sampling days.

At slaughterhouse two sample groups were considered:

1. pig samples: this group can be further subdivided into two groups:

   a) *Group 1*:

      - tonsils;
      - mesenteric lymph nodes;
      - colon content.

   This group indicates the pig status as an asymptomatic carrier of *Salmonella*. Tonsils are one of the first organs to come in contact with contaminated feedstuff or

faeces. Lymph nodes are less likely to be affected by contamination during transport and lairage compared to caecal contents, unless the time in transport and lairage is prolonged (e.g. more than 24 hours). Estimation of the prevalence of infected lymph nodes will therefore better reflect the status of the pig sent to slaughter than caecal contents (Vieira-Pinto et al., 2005; Nowak et al., 2007). Several studies demonstrated that tonsils, lymph nodes, faeces and the digestive tract are the most likely locations where *Salmonella* can be expected after contamination (Berends et al., 1996; Blaha et al., 2001; Swanenburg et al., 2001c; Nowak et al., 2007); in fact, according to Berends et al. (1996) the entire digestive tract, its contents and only the closely associated lymph nodes, such as the tonsils and mesenteric lymph nodes, have practical relevance as major sources of carcass contamination. On the same topic, Olsen et al. (2001) refers that, even when the slaughter process is performed correctly, the contamination from these tissues can be transferred to the carcass.

b) *Group 2*:

- carcass surface;
- liver surfaces.

This group can be considered as an indicator of the cross-contamination and consequently give information about hygiene during the slaughter process.

2. environment samples

Environmental samples were taken at different places.

a) scalding water;

b) contact surfaces with meat

- dehairing equipment;
- knives;
- carcass splitter:

c) any contact surfaces with meat:

- drain water;
- walls of the dirty zone.

S1, S2 and S5 were visited twice, S4 three times, S3 four times. At S2, S3, S4 and S5 both pig and environmental samples were taken. At S1 only pig samples were taken. Experiments took place during a whole sampling day.

MATERIALS AND METHODS

SAMPLES COLLECTION

Farm

1) Feed: an aliquot for each growing phase was sampled;

2) Faeces: from individual pigs, approximately 25 g of rectal content were taken and put in a sterile container. Samples were transported to the laboratory in a cooled container for immediate analysis.

Slaughterhouse

From individual pigs according to the Opinion of the EFSA on "Risk assessment and mitigation options of *Salmonella* in pig production" (2006), the following samples were taken:

1) carcasses surface: carcass sponge were taken, after evisceration, before chilling:

   - the upper inner part of both hind legs including approximately 5 cm of the skin and the pelvic entrance (approximately 30 cm x 20-25 cm);

- the cut surface area of the abdomen and chest including approximately 5 cm of the skin surface will be tested; approximately 70-80 cm x 8-10 cm shall be sampled.

A total area of approximately 1400 cm^2 of carcass surface was sampled. Two sterile sponge (10 x 10 cm) were used: one sponge for the upper inner part and one for the surface area of the abdomen.

Guts were collected in plastic bags, after evisceration of the pig and in a separate room near the slaughterline, samples of mesenteric lymph nodes were taken as follows:

2) mesenteric lymph nodes: according to the Opinion of the EFSA on "Risk assessment and mitigation options of *Salmonella* in pig production" (2006), at least 5 lymph nodes in the ileocaecal regions were cut out with a sterile, disposable scalpel;

Moreover, the following samples were taken:

1) colon content: with a sterile scalpel, the colon was incised and 25 g of colon content was collected in a sterile plastic stomacher bag;

2) liver: were sampled with sterile sponges over the surface on both sides; with the same sponge the liver was swabbed around all lobula. Sampling took place in the slaughter line immediately after removal of the pluck;

3) tonsils: were cut out and collected in plastic bags;

Environmental samples

The following samples were collected from the slaughterhouse environment, at the end of the sampling day, before cleaning:

1) scalding water: 25 ml were taken with a sterile collection tube. The temperature was measured;

2) any contact surfaces with meat: were sampled with the following procedures:

- drain water: at pre-chilling level, with sterile sponge;
- walls of the dirty zone: at stunning-bleeding level, a surface of 100 cm^2 was sampled with a sterile sponge.

3) contact surfaces with meat: were sampled with the following procedures:

- dehairing equipment: a surface of 1000 cm^2 with a sterile sponge;

- knives: from the tip to the base, twice;

- carcass splitter: on both sides of the blade, with a sterile sponge.

Sponges were immediately put in sterile plastic sponge-bags with a number on them. All samples were transported to the laboratory in a cooled container for immediate analysis.

In total 503 samples were taken, classified as follows:

- n.26 faeces at farm;

- n.15 feed at farm;

- n.85 pigs at slaughter;

- n.41 from slaughterhouse environment.

SALMONELLA ISOLATION METHOD

For the isolation of *Salmonella* the ISO method 6579/2002 modified according to EFSA report on "Risk assessment and mitigation opinion of *Salmonella* in pig production" was used.

Pre-enrichment

The pre-enrichment step was realized by using buffered peptone water (BPW, Unipath), as follows:

a) From each feed sample, 25 g was transferred into a stomacher bag and 225-ml BPW was added;

b) From the rectal contents/faeces samples, 25 g was transferred into a stomacher bag and 225-ml BPW was added.

c) Lymph nodes were put in boiling water for 3 s to eliminate superficial contamination, then were trimmed of any attached fat, meat or other tissue with

sterile material to keep the lymph node intact. Thereafter, 25 g of an intact lymph node was transferred in a stomacher bag with 225 ml BPW;

d) Tonsils were put in boiling water for 3 s to eliminate superficial contamination, thereafter cut into small pieces with sterile material. Then, 10 g was transferred in a stomacher bag with 90 ml BPW;

e) Sponge were suspended in 50 ml BPW;

f) From the water samples, 25 ml was transferred into a stomacher bag, and 225 ml BPW was added.

All the samples were homogeneized in a stomacher bag (BagPage) for 2 min and then incubated for 24 h at 37° C.

Enrichment

The pre-enrichment broth was mixed and 0.1 ml was transferred to 10 ml Rappaport–Vassiliadis soy broth (RV, Unipath) for 24 h at 41.5 °C. A loop of material from the RV broth was transferred and spread onto the surface of a xylose-lysine-tergitol4 agar plate (XLT4, Difco) so that isolated colonies could develop. The plates were incubated in inverted position at 37° C for 18–24 h. After incubation, the plates were checked for

growth of typical *Salmonella* colonies H2S-positive (black or black-centered with a yellow periphery).

The motility enrichment method with Modified Semisolid Rappaport Vassiliadis (MSRV, Unipath) was also used: three drops of 30 ml each of the pre-enrichment culture were transferred, in separate spots, onto a MSRV plate and incubated for 24 h at 42°C.

Selective plating

The plates were checked for migration zones with a radius larger than 10 mm. Negative plates were re-incubated for 24 h at 42°C. For migrated cultures, bacterial suspensions from the edge of the zone of migration were transferred onto the surface of both a Brilliant Green Agar plate (BGA, Unipath) and of a xylose lysine desoxycholate (XLD, Unipath) agar plate so that isolated colonies could develop. The plates were incubated in inverted position at 37 °C for 18–24 h. After incubation, the plates were checked for growth of typical *Salmonella* colonies whose morphology is the following:

- XLD: red-yellow colonies with or without black centers; degradation of xylose, lactose and sucrose generating acid products, cause color change in the medium from red to yellow;

- BGA: pink colonies, 1 mm to 2 mm in diameter, causing the colour of medium to change to red.

When typical colonies had grown, up to five colonies per plate were transferred and inoculated on triple sugar iron (TSI, Oxoid) agar slants which were incubated at 37 °C for 18–24 h with the cap tubes loosely in order to maintain aerobic conditions to prevent excessive H2S production. The reaction was considered positive when alkaline (red) slant and acid (yellow) butt, with or without production of H2S (blackening of agar) were detected.

IDENTIFICATION

The positive colonies were submitted to phenotypic identification with the ID 32E system (Biomerieux). For each isolate, the resulting 11-digit profile number was converted to an identification using APILAB Plus software (version 3.2.2).

SERO- AND PHAGE-TYPING

Isolated *Salmonella* strains were send to the laboratories of the "Centro Nazionale di Referenza per le salmonellosi" of Legnaro (Padua, Italy) for serotyping based on the antigenic structure of somatic (O), capsular (Vi) and flagellar (H) antigens. The antigens were detected using slide agglutination with commercially produced antisera, the O antigens using a suspension of growth from an agar plate while the H antigens using a suspension of broth culture.

The serotype was deduced from the specific pattern of agglutination reactions using the Kauffmann-White classification scheme.

Isolates belonging to serotypes Typhimurium and Enteritidis were submitted to phage typing based on specific bacteriophage lysing bacteria. Very dry agar plates containing a rich nutrient source, were flooded with a liquid culture of the bacterial isolate. Then, the liquid culture was removed, the culture film allowed to dry, and a set of phage suspensions were inoculated onto the surface of the plate by means of a multipoint inoculator. Phage spots were left to dry, and plates were incubated inverted overnight at

37°C. The next day, phage lysis reactions were recorded, and compared to a set of standards developed by Public Health Laboratory Service (PHLS), Colindale, England.

A further part of the study has been conducted in the period April-August 2008 next to the Scottish *Salmonella* Reference laboratories, placed at Stobhill Hospital (Glasgow), with the following aims:

- Analyse the isolates for their susceptibility to various antibiotics;
- Investigate clonal diversity of the isolates with the following methods:
  - Pulsed field gel electrophoresis (PFGE);
  - Plasmid profile analysis (PPA);
- Analyse the isolates for the presence of antibiotic resistance genes;
- Genotypic characterization microbial resistance genes;
- Plasmid transfer assay.

PFGE

All the *Salmonella* isolates were inoculated onto cysteine lactose electrolyte deficient (CLED, Oxoid) and incubated overnight at 37°C to evaluate the purity. A control strain of *S.* Braenderup, to serve as a molecular weight standard, was also inoculated onto CLED. For chromosomal DNA preparation for subsequent PFGE, the SalmGene protocol by Peters et al. (2003) was used. *Salmonella* isolates were inoculated onto 5 ml brain hearth infusion broth (BHI, Oxoid) in a universal container and incubated overnight at 37°C. 1 ml of culture was transferred into a sterile Eppendorf tube and centrifuged at 13,000rpm for 3 minutes. The supernatant was carefully removed and the cells were resuspended in 1 ml of sterile saline (0.85% w/v NaCl) and centrifuged at 13,000 rpm for 3 minutes. Again, the supernatant was discarded and the cells were resuspended in 500 µl of saline (turbidity of McFarland standard 5.0). The tubes were transferred to a heating block (Stuart SBH130DC) at 40°C to equilibrate. Agarose plugs were prepared as follows: 2% of agarose (Pulsed Field Certified Agarose, Biorad) was dissolved in saline by gentle heating in a microwave (0,5ml per sample) and transferred

to a heated waterbath (56°C) to equilibrate. Plugs moulds were prepared by sealing the underside with tape to prevent leakage. 500 µl of agarose were added to the resuspended cells, mixed thoroughly and immediately dispensed into the wells of the plug moulds. Wells were slightly overfilled to allow for shrinkage of cooled agarose (about 150 µl per well). Moulds were placed at 4°C for 15-20 minutes to facilitate setting of the agarose. For the cell lysis 1 ml of ES buffer pH 9.5, (0.5 M EDTA, 1% N-lauroylsarcosline) (Sarkosyl NL-30) dissolved in milliQ water, was dispensed into labelled Eppendorf tubes and 20µl of Proteinase K (50mg/ml, Sigma P-0390) were added. The plugs were gently pushed into the ES buffer and incubated overnight in a heated waterbath (56°C) to allow cell lysis. Following lysis the plugs were transferred into a labelled 5ml plastic tube containing 3 ml of TE (PFGE) buffer (pH 7.5) (10mM Tris methylamine, 10mM EDTA), and incubated in shaking waterbath at 54 °C for 15-20 minutes. This stage was repeated at least 5-6 times to thoroughly wash the plugs. For the restriction endonuclease digestion of DNA, 100 µl per sample of 1x reaction buffer (supplied with enzyme by manufacturer) were dispensed into labelled Eppendorf tubes. A thin slice (about 1mm) was cut from across the width of the agarose plug with a clean scalpel blade onto the surface of a clean Petri plate, placed in the reaction buffer and

incubated at room temperature for 30-60 minutes. Again, 100µl per sample of 1x reaction buffer were dispensed into a sterile plastic universal and 50 Units per sample of XbaI (10U/µl, Invitrogen) were added. The reaction buffer in which the plugs had been incubated, was replaced with 100µl of fresh buffer plus enzyme and incubated at 37°C for 4 hours. An agarose gel was prepared as follows: agarose 1% (BioRad Pulsed Field Certified) was dissolved by heating in 0.5X TBE buffer consisting in 50mM Tris, 45 mM Boric Acid and 0.5mM EDTA, allowed to cool to 50°C, poured on a tray and allow to set for at least 30 minutes. Once set, the gel was cooled to 4°C. Approximately 2 ml of gel were retained to seal the wells. The plugs were inserted into the wells, ensuring that they touched the front wall of the well and that there were not air bubbles present. Size markers were loaded in the same manner. The plugs were sealed into the wells by using the reserved agarose and allow to set before placing the gel into the electrophoresis tank.

PFGE was performed with CHEF DRII system (Bio-Rad). The tank was filled with 2.25 litres of 0.5X TBE buffer and chilled to 13°C before loading. Electrophoresis conditions were 6V/cm for 22 h. Pulse time was ramped from 2 to 64 s. The gel was stained with ethidium bromide and photographed under UV illumination.

Macrorestriction patterns were compared with the use of Bionumerics software (Applied Maths, Belgium). The molecular weights of the restriction fragments were calculated by comparison with the external markers, and images were normalized accordingly. Different profiles were assigned to types in accordance with differences in the restriction patterns.

PPA

Plasmid DNA was isolated by the alkaline lysis method described by Kado and Liu (Kado and Liu, 1981).

All the *Salmonella* isolates were inoculated onto cysteine lactose electrolyte deficient (CLED, Oxoid) and incubated overnight at 37°C to evaluate purity. The strains were then inoculated into 5 ml brain hearth infusion broth (BHI, Oxoid) in a universal container and incubated overnight at 37°C. 800 µl were transferred to a sterile Eppendorf tube and centrifuged (Biofuge Pico Haeraeus) at 13,000 rpm for 5 minutes. The supernatant fluid was discarded, the cell pellet was resuspended in 20 µl of TE 50:1 buffer (50mM Tris, 1mM EDTA) using a vortex mixer (Vortex Genie 2, Scientific

Industries). 100 µl of Kado and Liu lysis buffer consisting of 3%SDS in 50mM Tris (pH 12.6) were added and the content gently mixed by inversion. The tubes were incubated at 56°C for 30 minutes in a heated water bath (Luckham WB150). Then, 100 µl of phenol:choloform:isoamyl alcohol (in the proportion of 25:24:1, Sigma Aldrich) were added, vortexed vigorously until the suspension was a homogeneous milk-white and centrifuged for 15 minutes at 13,000 rpm. Then, 40 µl were carefully removed from the upper aqueous phase and transferred to a clean Eppendorf tube. 6 µl of tracking dye (Gel Loading Buffer, Sigma Aldrich) were added to each tube. To estimate plasmid sizes *E.coli* 39R861 was used as a molecular weight marker: a mixture of 39R861 plasmid DNA (40 µl of for each gel to be run) and Supercoiled Ladder (Sigma Aldrich) was prepared by adding 1.5 µl of ladder to 40µl of 39R861 and 6µl of tracking dye. 25 µl of this mixture was applied to each of the outside wells of an agarose gel consisting in 0.7 g of agarose (General Purpose Agarose, Mast Diagnostics) dissolved by heating in 100 ml of TBE 1X buffer. 25 µl of each test sample were loaded onto the gel. A tank (Flowgen) was filled with Kado and Liu lysis buffer and the gel was electrophoresed at the constant voltage of 100V for three hours. Following electrophoresis it was stained

with ethidium bromide, rinsed in distilled water and photographed under UV illumination.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

The MICs of the *Salmonella* isolates were determined according to the National Committee for Clinical Laboratory Standard guidelines for the agar dilution method. The following antimicrobials were tested:

- Ampicillin;
- Choloramphenicol;
- Chloramphenicol;
- Gentamicin;
- Nalidixic acid;
- Streptomycin;
- Tetracycline;
- Trimethoprim;
- Cephalothin;

- Sulphamethoxazole.

Isosensitest agar (Oxoid) plates were used: for each 200 ml of agar, approximately 25 ml were distributed into a sterile Petri-dish and allowed to set. A set of plates containing one of the listed antibiotics, except for sulphamethoxazole, plus a control plate with no antibiotic, were inoculated with the organisms to be tested. For sulphamethoxazole Mueller Hinton agar (Oxoid) was used in the same proportion as Isosensitest agar (table 1). Amoxicillin-clavulanic acid, colistin and cefotaxime were evaluated by Oxoid disk susceptibility testing on Mueller Hinton agar plates according to the recommendations of the National Committee for Clinical Laboratory Standards (table 2). Previous to the analysis, the strains to be tested were inoculated onto CLED agar plates and incubated overnight at 37°C. A single colony from each plate was inoculated into 3 ml of Tryptone water (Oxoid) and incubated for three hours at 37°C. 30µl of tryptone water were added to 3ml of sterile water and 0,5 ml of this solution was immediately transferred to a well of a 16-welled Perspex plate using a multipoint inoculator 16 loops: loops were dipped in alcohol, flamed, allowed to cool, charged with organism then inoculated onto the plates. All the plates were incubated at 37°C overnight. Growth was recorded as resistant and no growth as sensitive. As regard to the disk susceptibility

testing, isolates to be tested were inoculated with a swab onto Mueller Hinton agar plates and then the disks were applied. The plates were incubated overnight at 37°C. The zone diameters were measured and recorded.

PREPARATION OF TEMPLATE DNA

Organisms that showed antibiotic resistance were inoculated onto CLED and incubated at 37°C overnight. Three colonies from each plate were inoculated into a 1.5ml sterile Eppendorf which contained 100µl of sterile water. The tubes were boiled in a block heater at 105°C for twelve minutes, then centrifuged at 13,000rpm for ten minutes. 80µl of the supernatant were transferred to a new sterile Eppendorf and stored at -20°C indefinitely.

PCR AMPLIFICATION

The oligonucleotide primers for antibiotic resistance genes and for class 1 integrons are shown in table 3. Primers were synthesized by VH Bio Ltd, Gateshead, UK. First, by using the 5'-CS and the 3'-CS primers, the variable regions of the integrons was amplified. Then, we detected the class 1 integrase-specific *Int-1* gene. Finally we investigated the presence of the resistance genes.

All PCR amplifications were carried out by using puReTaq Ready-To-Go PCR beads (GE Healthcare) which contained pre-dispensed Taq polymerase, nucleotide and buffer, in accordance to the manufacturer's instruction. PCR amplifications were performed in a 25µl volume, using a GeneAmp 9700 Thermocycler (Applied Biosystems). Reaction mixtures contained 2.5µl of each primer (from stock concentration of 10pmol/µl), 5 µl of template DNA and sterile water. The cycling parameters for each primer are shown in table 4. Int1 was run on a 1% agarose gel, CS was run in an 1.6% agarose gel and the other oligonucleotide primers in a 1.5% agarose gel. Gels were electrophoresed at 90V for 1 hour. All the gels were stained with ethidium bromide (0.5 µg/mL) and photographed under UV illumination. PCR product sizes were estimated by comparison with a 100-bp ladder (Thermo Scientific).

PLASMID TRANSFER ASSAY

We performed conjugation experiments to determine whether antimicrobial resistance markers were located on conjugative plasmids, on n.3 isolates that had showed resistance to the following antibiotics:

n.1 and n.2: ACSuTTmKf;

n.3: ACSuTKfAx.

Prior to the analysis both the donor and the recipient were inoculated onto CLED and incubated overnight at 37°C to check purity and obtain a fresh colony.

Mating process

Candidate MDR donor strains were mated with a spontaneous rifampicin resistant *E.coli* J53-2 strain. The isolates and the donor were grown in 10 ml of BHI broth (Oxoid) in a universal container and incubated overnight at 37°C. Thereafter, 200 µl of the donor were mixed with 800 µl of the recipient, 4 ml of BHI were added and cells were harvested by centrifugation at 3000 rpm and the mixture was incubated at 37°C for 4 h.

Selection of transconjugant

Transconjugant were selected on CLED agar plates containing one of the antibiotic to be tested (see table 1 for concentrations), and on CLED agar plates containing rifampicin. These plates were inoculated with 100µl of the mating mixture using a sterile spreader.

Control plates, also containing both one of the antibiotic to be tested and rifampicin were inoculated from both cultures of the donor, recipient and mating mixture to ensure that:

- the organisms were resistant to the appropriate antibiotic;
- both donor and recipient were present in the mating mixture.

All the plates were incubated overnight at 37°C.

The following day potential transconjugant were purified and confirmed to be *E.coli* by growth onto CLED.

RESULTS AND DISCUSSION

SALMONELLA PREVALENCE AND SEROTYPES

Salmonella could be isolated from 64 of 425 samples of slaughtered pigs (15%) and t from 13 of 41 environmental samples (31.8%).

Salmonella could not be isolated in any of the samples taken at farm.

The prevalence of *Salmonella* differed between slaughterhouses and, within slaughterhouses, between sampling days (SD).

In general, among slaughtered pigs samples, mesenteric lymph nodes resulted in the highest prevalence (30.5% or 26/85), followed by colon content (16.4% or 14/85), carcass and liver (both 14.1% or 12/85).

Salmonella could not be detected in any of the tonsil samples.

The prevalence of *Salmonella* in all pig samples was 36% in slaughterhouse *S4*, 13.3% in *S1*, 12% in *S3* and 3.8 % in *S5*. *Salmonella* was not detected in any of the pig samples taken in *S2*.

In slaughterhouse *S1 Salmonella* could be detected only in lymph nodes during both SD. In slaughterhouse *S5 Salmonella* could be detected only in the samples from colon content. In slaughterhouses *S4* and *S3, Salmonella* was detected in all pig sample types, even though with different prevalence.

Figure 1 shows the *Salmonella* prevalence per slaughterhouse and pig samples.

The prevalence of pigs with *Salmonella* in one or more samples was 42.3%.

N. 31 pigs were carriers of *Salmonella* in lymph nodes and/or colon content. In n.8 pigs among these subjects, *Salmonella* could be isolated in the carcass too.

Salmonella prevalence was slightly higher in any contact surfaces samples (37.5%) than in contact surfaces samples (35.2%). *Salmonella* was not detected in any of the environmental samples taken in slaughterhouse *S2*. In *S3 Salmonella* could be isolated in all the samples of drain water, in one sample of dehairing equipment (20%) and in

one sample of carcass splitter equipment (20%). In *S4 Salmonella* could be detected in all environmental samples, including one sample of scalding water.

Figure 2 shows the *Salmonella* prevalence per slaughterhouse in environmental samples.

Table 5, 6, 7 and 8 show the *Salmonella* prevalence per slaughterhouse and SD in the pig and environment samples.

In total n.103 *Salmonella* strains were isolated, classified in 8 serotypes.

The most frequently *Salmonella* serotype was Derby (43%), followed by Typhimurium (23%) and Panama (18%); other serotypes isolated were Livingstone (5%), Infantis (5%) and Rissen (4%). Newport and Bredeney serotype were rarely isolated (1%).

Figure 3 shows the prevalence of *Salmonella* serotypes.

Phage typing of the *S. typhimurium* isolates resulted into 4 different phage types: DT193 (n.12 strains), DT59 (n.4), DT104B (n.1), U302 (n.1); 6 Typhimurium strains were untypable.

Figure 4 shows the prevalence of *S. typhimurium* phage types.

Among the n.87 strains from pig samples, all the serotypes were isolated. *S. derby* resulted in the highest prevalence (43.6%).

On the contrary, the n.16 strains from environmental samples belonged to only two serotypes. *S. typhimurium* showed the highest prevalence (62.5%) respect to Derby.

Table 9 shows the *Salmonella* serotype distribution in pig and environmental samples.

Table 10 shows *Salmonella* serotypes distribution per slaughterhouse in pig samples; table 11 shows *Salmonella* serotypes distribution per slaughterhouse in environmental samples. Table 12 shows the detail of the *Salmonella* serotypes isolated from the pig and environmental samples per slaughterhouse and per SD. The SD are indicated as I, II, III and IV preceded by the slaughterhouse abbreviation.

A different distribution of the serotypes was observed in slaughterhouse S3, in relation to the SD (table 12). During SD 1 *S. typhimurium* 193 was isolated from both a lymph node and a colon content sample of the same pig. During SD 4 *S. panama* could be isolated from samples of carcass, colon content and liver of one pig, and from both colon content and liver of other two animals. During the same SD *S. typhimurium* untypable was isolated from a carcass and a drain water sample. In spite of the lack of

homogeneity, probably due to the different origin (regional and European) of the slaughtered pigs, the results emphasize the importance of the cross-contamination between contiguous carcasses of carriers and the environment.

A different situation was detected in slaughterhouse S4. During SD 2 *S. derby* was isolated from all the pig samples types and from drain water samples. Moreover, during SD 3 all the same samples were contaminated and the same serotype was isolated from dehairing equipment and scalding water samples. During SD4 it was detected in a sample of splitting equipment.

DISCUSSION

The results of our survey show that the prevalence of slaughtered pigs contaminated with *Salmonella* can be rather high. In 41 % of the sampled pigs *Salmonella* was observed at one or more sampling sites, according to other similar studies (Swanenburg et al., 2001c; Botteldoorn et al., 2003).

The presence of *Salmonella* carriers (n. 31) is one of the factors affecting the final carcasses contamination (Bonardi et al., 2003). As many authors indicate, slaughter equipment is cause of cross-contamination (Swanenburg et al., 2001a and c; Botteldoorn et al., 2003).

Salmonella serotypes, that can survive in certain niches of the slaughterhouse environment, can become part of the resident flora (house strains). As a consequence, slaughtered pigs can get contaminated.

As said, *Salmonella* was isolated from one water sample of the scalding tank in slaughterhouse S4. This indicates that the water temperature was not always above 60 °C or that *Salmonella* embedded in organic material, and consequently protected against high temperature (Swanenburg et al., 2001c).

Further, *Salmonella* was isolated from the drain water in all the samples taken at slaughterhouse S3 and S4. However, from this sampling site in S3 three different serotypes were isolated during three distinct SD, while in S4 the same serotype was isolated during two SD. Usually, the contaminated drain water is not considered as a critical point, but the carcasse contamination can occur if water from the drains is

spread out during cleaning procedures, e.g. under high pressure. The increasing prevalence of *Salmonella* in the drain water samples was probably correlated to inadequate application of cleaning procedures, that allow the persistence of house strains biofilm producer (Malcova, 2008). It cannot be excluded that *Salmonella* was able to multiply in the drains (Swanenburg et al., 2001c).

Finally, in general the *Salmonella* presence in the slaughterhouse environment depend on the microbial flora carried by the slaughtered pigs during the same day. The different prevalence observed between SD and slaughterhouses can depend on the Slaughtering and Hygienic Practices application by the workers, the moment of the sampling and the origin of the animals.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antimicrobial resistance

All the 103 isolates were susceptible to ciprofloxacin, gentamicin, nalidixic acid, cefotaxime and colistin. Of all the isolates tested 71 (69 %) were susceptible to all antimicrobial tested. The remaining 32 isolates (31 %) were found resistant to at least one antibiotic (table 13). The most common resistance observed was to cefalothin (100%), followed by ampicillin (90.7 %), tetracycline (65.7 %), sulphametaxole (62.5 %), amoxiclav (53.1 %), cloramphenicol (34.4 %), trimethoprim (28.2 %) and streptomycin (22%).

Antimicrobial resistance (AMR) was found among the following serotypes: S. typhimurium (95.6 %), S. derby (18.2 %) and S. rissen (25 %)

Table 13 shows the AMR prevalence among *Salmonella* serotypes.

Among the 32 isolates that showed resistance 30 (93.8%) were found to be resistant to more than one antimicrobial.

Multidrug resistance (MDR) profiles

All the *S. typhimurium* isolates that showed resistance were resistant to at least 3 antimicrobial.

Among *S. typhimurium* isolates four different MDR patterns were found:

- ACSuTKfAx (6 isolates);

- ASSuTTmKf (2 isolates);

- ASSuTKf (1 isolate);

- ACSuTTmKf (1 isolate).

Among MDR *S. typhimurium* strains, n. 10 were isolated from environmental samples, n. 9 from colon content.

Among S. derby two different MDR patterns were found:

- ACSuTTmKf (4 isolates);

-ASuTTmKFAx (2 isolates).

All the MDR S. derby were isolated from lymph nodes samples.

DISCUSSION

Our results about AMR prevalence in *Salmonella* swine isolates are in contrast with other studies carried out at farm level (Gebreyes et al., 2004; Aragaw et al., 2007; Garcia-Feliz et al., 2008; Perron et al., 2008) and at slaughter (Davies et al., 2004), where the most common resistance phenotypes to tetracycline was found.

In general a high level of resistance was observed to frequently used antimicrobials in veterinary and public health practices, including streptomycin and tetracycline, as it might be expected (Aragaw et al., 2007).

We detected a different resistance to amynoglicosides: the overall resistance to ampicillin was high (90.7%), while resistance to amoxiclav was 53.1%, to streptomycin 22% and no isolate showed resistance to gentamicin. Antimicrobials within the same class usually possess slight structural differences to minimize cross-resistance (Farrington et al., 2001).

However, we have also observed resistance to antimicrobials that are not commonly used in animal health and production in Europe, such as ampicillin, ciprofloxacin, nalidixic acid, chloramphenicol, florfenicol, gentamicin. This suggests the importance

of other sources of MDR resistant *Salmonella* serovars, such as imported contaminated items, use of the antimicrobials in the human health sector and possibly cross resistance among the related antimicrobials.

DETECTION OF CLASS 1 INTEGRONS BY PCR

Heterogeneous integron-associated resistance genes were present in the isolates under study (table 15).

There was not always a good correlation between the presence of resistance genes and corresponding resistance phenotypes.

All the strains that were streptomycin-resistant contained both the gene *aadA1* and *aadA2*; all the strains that were tetracycline-resistant contained the gene *tetA(G)* and either the gene *tetA(A)* or *tetA(B)*.

However, there were several cases in which a strain was resistant to an antibiotic but the identity of the gene conferring resistance was not ascribed. None of the strains that were ampicillin-resistant contained the gene *bla(carb$_2$)* and 8 of 30 strains did not contain the *bla(tem)* gene. 7 of 11 cloramphenicol-resistant strains contained the *cat1* gene and

none of them the *cat2* gene. Just 4 of 15 strains that where trimethoprim-resistant contained the *dhfr1* gene.

PLASMID PROFILE ANALYSIS (PPA)

Sixtysix of 103 strains (64%) were found to harbour at least one plasmid (Table 14). Eigth strains were found to carry five plasmids, n. 11 three plasmids, n. 3 two plasmids and n. 44 one plasmid. The molecular weight ranged from 3.3 to 130 kb.

The remaining 37 strains (36%) were plasmid free.

The following *Salmonella* serotypes were found to harbour one or more plasmids: Derby, Typhimurium, Livingstone and Rissen.

All the isolates of *S. derby* carried at least one plasmid: n. 10 strains (23%) had three plamids, n. 1 strain (3%) two plasmids and n. 33 (75%) had one plasmid; 6 plasmid profiles (PP) were identified. Five *S. derby* strains, isolated from lymph nodes samples of five different animals in slaughterhouse S1 during the same sampling day, carried two different plasmids of ca. 90 and 3.6 kb, but one was sensitive to all the antimicrobials tested; the other four harboured an additional large plasmid of ca. 180 kb and showed the MDR profile ACSuTTmKf.

The same situation was detected among *S. livingstone* isolates: one isolate harboured a plasmid of ca. 85 kb, while other four strains, isolated from lymph nodes samples of

five different animals in slaughterhouse S1 during the same sampling day, were plasmid free. In this case however, all the strains were fully sensitive to the antimicrobials tested.

Twenty (83.4%) *S. typhimurium* strains carried at least one plasmid: n. 9 harboured one plasmid, n. 2 two plasmids, n. 1 three plasmids and n. 8 five plasmids.

PFGE ANALYSIS

The application of XbaI macrorestriction to the 103 *Salmonella* strains produced n. 20 distinct profiles (table 14 and figure 5).

Within the different serotypes different pulsetypes were observed:

- *S. derby* (n. 44): 4 (DerX 21-22-23-24);
- *S. typhimurium* (n. 23): 7 (STYMXB.0046; STYMXB.0051; STYMXB.0058; STYMXB.0079like; Tm120X15; Tm193X13; Tmunt16);
- *S. infantis* (n.5): 3 (SINFXB.0014; SINFXB.0039; SINFXB.0074);
- *S. rissen* (n.4): 2 (RisX9; RisX10);
- *S. livingstone* (n. 5): 1 (LivX15);
- *S. panama* (n. 19):1 (PanX18);
- *S. bredeney* (n.1): 1 (BreX16);
- *S. newport* (n.1): 1 (NewX70).

Between different slaughterhouses common pulsetypes have not been observed, while in some cases different pulsetypes of the same serotype have been identified within the same slaughterhouse.

Distribution of S.derby and S. tiphymurium pulsetypes per slaughterhouse and per sampling days

- ***S. derby***

In S1: during the SD I, two pulsetypes (4 designated as DerX21 and 1 DerX23) have been detected in 5 lymph nodes from 5 pigs. The variation of DerX23 was represented by the absence of a 180-kb fragment representing a large plasmid. Moreover, these features were linked with the presence-absence of MDR, suggesting that the AMR markers could be located on conjugative plasmid.

In S3: during the same SD (III) the same pulsetype (DerX24) was isolated from two lymph nodes of 2 pigs.

In S4: the same pulsetype (DerX22) has been isolated during two SD, from all pig samples (SD II and III), and from all the environmental samples (SD II) and from splitting equipment and drain water (SD III).

- ***S. typhimurium***

S. typhimurium was isolated only in S3, during 4 different SD, and in S4, during 1 SD. However it was no possible to isolate the same pulse-type within the same slaughterhouse in different sampling days.

In S3: during the SD I STYMXB.0051 pulsetype was isolated from two colon content samples; during the SD II STYMXB.0058 was isolated from 1 sample of drain water, and Tm120X15 from 1 liver sample; during the SD III STYMXB.0079like was isolated from all environmental samples; during the SD IV Tmunt16 was isolated from 3 carcasses and from drain water samples.

In S4: during the SD I only Tm193X13 pulse-type was isolated from one lymph nodes.

In S5: during the SD I STYMXB.0046 pulsotype was isolated from 7 colon content and 2 splitting equipment samples.

DISCUSSION

The results of PFGE analysis improve the epidemiological survey about *Salmonella* traceability in Sardinian pig slaughterhouses. A large variability of *Salmonella* population at pig abattoir as been confirmed. Moreover the detection of the same S. derby pulsetype (DerX22) within the same plant, during two SD, from both pig and environmental samples, suggest that the strain has become house flora, therefore indicating a possible source of cross-contamination.

The combinated application of PPA and PFGE gave additional useful information about *Salmonella* routes in pig slaughterhouses.

PLASMID TRANSFER

The conjugation experiment showed that none of the multidrug-resistant *Salmonella spp.* tested could transfer its antimicrobial resistance to the *E.coli* J53-2 recipient.

CONCLUSIONS

Our survey give interesting information about the *Salmonella* prevalence in Sardinian slaughterhouses and the sources of direct and cross-contamination by *Salmonella* of swine meat.

Salmonella could be isolated from 15% of slaughtered pig samples and from 31.8% of environmental samples. Mesenteric lymph nodes resulted in the highest prevalence (30.5%). These results are in accordance with other similar studies (Swanenburg et al., 2001c; Botteldoorn et al., 2003). In 41 % of the sampled pigs *Salmonella* was observed at one or more sampling sites, which represents a potential increase of the risk.

As it was shown, many salmonellae were brought into the slaughterhouses by the delivered pigs. In fact, n. 31 pigs were carriers of *Salmonella* in lymph nodes and/or colon content. In n.8 pigs among these subjects, *Salmonella* could be isolated in the carcass too. For the interpretation of our data, it has to be kept in mind that the carrier pigs (with positive colon content and/or mesenteric lymph nodes) in the slaughterhouse could have been infected on the farm and during transport or during the waiting period

in the lairage before slaughtering. Several authors indicates that the contamination can already be detected in the faeces and the mesenteric lymph nodes as early as 3 h after infection (Fedorka-Cray et al. 1994). Especially the lairage and the high contamination level of the slaughterhouse environment are probably the major source for *Salmonella* infections prior to slaughter (Hurd et al. 2001; Swanenburg et al. 2001b). However, it's not always easy to correlate the role of the carriers with the carcass contamination.

The most prevalent serotypes detected were *S. derby* and *S. typhimurium*; these results are in accordance with other similar studies carried out both in pig farms (Weigel et al., 2007) and at pig slaughterhouses (Nollet et al., 2004). It is important to remind that all the serotypes associated with swine species are considered potentially dangerous for public health and cases of non typhoid salmonellosis infections have been described in humans.

The prevalence of *S. typhimurium* DT 193 (50%) is of a certain importance, since such phage type has been associated with foodborne outbreaks (in Italy and the United Kingdom) traced to contaminated pork products (Gebreyes et al., 2002).

During our survey, in three slaughterhouses (S3, S4 and S5) it was possible to isolate the same serotypes both from pig and environmental samples. In S4 *S. derby* was

isolated from pig and environmental samples during two different SD, suggesting that the strain had become house flora, therefore indicating a possible source of cross-contamination. The application of PFGE strongly confirmed these results, since in this case it was possible to isolate only one *S. derby* pulsetype (DerX22). In S3 the situation was less homogeneous, since it was not possible to isolate the same *S. typhimurium* serotype and pulsetype between different SD; this heterogeneity was probably due to the fact that the slaughtered pigs were both Sardinian and imported from EC.

The combinated application of PPA and PFGE gave additional useful information about the characterization of *Salmonella* strains.

The most common AMR observed was to cefalothin (100%), followed by ampicillin (90.7 %), tetracycline (65.7 %), sulphametaxole (62.5 %), amoxiclav (53.1 %), cloramphenicol (34.4 %), trimethoprim (28.2 %) and streptomycin (22%). These results are in contrast with other studies carried out at farm level (Gebreyes et al., 2004; Molla et al., 2006; Garcia-Feliz et al., 2007; Perron et al., 2008) and at slaughter (Davies et al., 2004), where the most common resistance phenotypes to tetracycline was found. MDR was found in association with serotypes Derby and Typhimurium: the increasing

multiple antimicrobial resistance associated with such pork-related serotypes may become a serious human health hazard in the near future (Boyen et al., 2008).

In our study it was not possible to detect resistance to fluoroquinolones and cephalosporins, which represents a favourable situation with regard to public health. However, the detection of a limited number of resistant isolates to ciprofloxacin emphasizes the importance of monitoring antimicrobial resistance among *Salmonella* isolates of porcine origin in order to detect new and emerging resistance trends.

Our survey was carried out in cooperation with the food business operators (FBO) and the Public Health Veterinary Service and our results have been useful to conform their standards with the microbiological criteria set out in Commission Regulation (EC) No 2073/2005 and No 1441/2007. In case of unsatisfactory results the actions to be taken by the FBO consist in improvement in slaughter hygiene and review of process controls. Moreover, the identification of origin of infected animals and the application of the biosecurity measures in the farms of origin should be encouraged. The FBO could decide to introduce in the slaughterhouse only animals from certified *Salmonella*-negative farms or slaughter separately animals from positive and negative herds.

A residential *Salmonella* flora should not be present in the slaughterhouse. Therefore, some preventive measures should be encouraged. Slaughter should be performed according to HACCP principles in association with good hygiene procedures (GHP), in order to avoid faecal contamination of carcasses by increasing care during evisceration, and alterating the meat inspection procedures. Regular cleaning and disinfecting of all equipment, also during slaughter, should be carried out; results of cleaning and disinfecting should be checked.

TABLES AND FIGURES

Table 1. Antibiotics and plate concentration per milliliter used for AMR determination of *Salmonella* strains.

Antibiotic	Abbreviation	Concentration µg/ml
Ampicillin	**A**	50
Chloramphenicol	**C**	20
Ciprofloxacin (0.5)	**Cp**	0.5
Gentamicin	**Gm**	20
Nalidixic acid	**Na**	40
Streptomycin	**St**	20
Sulphametaxole	**Su**	100
Tetracycline	**T**	10
Trimethoprim	**Tm**	10
Cefotaxime	**Cx**	1

Table 2. Antibiotics and disk concentration used for AMR determination of *Salmonella* strains.

Antibiotic	Abbreviation	Disk content
Colistin	**Co**	25
Cephalotin	**Kf**	30
Amoxicillin-clavulanic acid	**Ax**	20/10

Table 3. Primer sequences for antibiotic resistance genes and for class 1 integrons.

Gene of interest	F or R	5′ to 3′ DNA sequence of primers	Reference
Int1	F	TCTCGGGTAACATCAAGG	Leverstein et al., 2002
	R	AGGAGATCCGAAGACCTC	
CS	F	GGCATCCAAGCAGCAAGC	Levesque et al., 1995
	R	AAGCAGACTTGACCTGAT	
aadA1	F	TATCAGAGGTAGTTGGCGTCAT	Randall et al., 2004
	R	GTTCCATAGCGTTAAGGTTTCATT	
aadA2	F	TGTTGGTTACTGTGGCCGTA	Randall et al., 2004
	R	GATCTCGCCTTTCACAAAGC	
bla (Tem)	F	CATTTCCGTGTCGCCCTTAT	Randall et al., 2004
	R	TCCATAGTTGCCTGACTCCC	
bla ($Carb_2$)	F	GCTTCGCAACTATGACTAC	Randall et al., 2004
	R	GTTCACCATCCAAGACTC	
cat1	F	CCTATAACCAGACCGTTCAG	Randall et al., 2004
	R	TCACAGACGGCATGATGAAC	
cat2	F	CCGGATTGACCTGAATACCT	Randall et al., 2004
	R	TCACATACTGCATGATGAAC	
dhfr1	F	GTGAAACTATCACTAATGGTAGCT	Randall et al., 2004
	R	ACCCTTTTGCCAGATTTGGTAACT	
sul1	F	TCACCGAGGACTCCTTCTTC	Randall et al., 2004
	R	AATATCGGGATAGAGCGCAG	
tetA(A)	F	GGTTCACTCGAACGACGTCA	Randall et al., 2004
	R	CTGTCCGACAAGTTGCATGA	
tetA(B)	F	CCTCAGCTTCTCAACGCGTG	Randall et al., 2004
	R	GCACCTTGCTCATGACTCTT	
tetA(G)	F	CCGGTCTTATGGGTGCTCTA	Randall et al., 2004
	R	CCAGAAGAACGAAGCCAGTC	

Table 4. Cycling parameters of PCR amplification of MDR *Salmonella* strains.

GENE OF INTEREST	CYCLING PARAMETERS			REFERENCE
	DENATURATION[1]	ANNEALING	EXTENSION[2]	
Int1	94°C (45 sec)	54°C (30 sec)	72°C (1 min)	Leverstein-van-Hall et al., 2002
5'-CS	94°C (45 sec)	53°C (30 sec)	72°C (1 min)	Levesque et al., 1995
aadA1	94°C (1 min)	54°C (1 min)	72°C (1 min)	Randall et al., 2004
aadA2	94°C(1 min)	60°C (1 min)	72°C (1 min)	Randall et al., 2004
bla($Carb_2$)	94°C (45 sec)	52°C (1 min)	72°C (50 sec)	Randall et al., 2004
bla(Tem)	94°C(1 min)	54°C (1 min)	72°C (1 min)	Randall et al., 2004
cat1	94°C (1 min)	56°C (1 min)	72°C (1 min)	Randall et al., 2004
cat2	94°C (1 min)	56°C (1 min)	72°C (1 min)	Randall et al., 2004
dhfr1	94°C (1 min)	54°C (1 min)	72°C (1 min)	Randall et al., 2004
sul1	94°C (1 min)	60°C (1 min)	72°C (1 min)	Randall et al., 2004
sul2	94°C (1 min)	64°C (1 min)	72°C (1 min)	Randall et al., 2004
tetA(A)	94°C (1 min)	56°C (1 min)	72°C (1 min)	Randall et al., 2004
tetA(B)	94°C (1 min)	56°C (1 min)	72°C (1 min)	Randall et al., 2004
tetA(G)	94°C (1 min)	60°C (1 min)	72°C (1 min)	Randall et al., 2004

[1] Five minute for the first cycle

[2] Seven minutes for the last cycle

Table 5. Prevalence (%) of *Salmonella* per sample type and sampling day in *S1*

	Prevalence (Pos/N)		
	Total	Sampling Day 1	Sampling Day 2
Pig Samples			
Lymph nodes	66.6	100 (5/5)	50 (5/10)
Colon content	0	0	0
Carcass sponge	0	0	0
Liver sponge	0	0	0
Tonsils	0	0	0
Total	13.3	14.2	10
Environment samples			
Contact surfaces with meat	nd	nd	nd
Not contact surfaces with meat	nd	nd	nd
Scalding water	nd	nd	nd
Total	nd	nd	nd

nd: not done

Table 6. Prevalence (%) of *Salmonella* per sample type and sampling day in *S3.*

	Prevalence (Pos/N)				
	Total	Sampling Day 1	Sampling Day 2	Sampling Day 3	Sampling Day 4
Pig samples					
Lymph nodes	16	20 (2/10)	0	40 (2/5)	0
Colon content	8	10 (1/10)	0	0	20(1/5)
Carcass sponge	20	0	0	0	100 (5/5)
Liver sponge	16	0	20 (1/5)	0	60 (3/5)
Tonsils	0	0	0	0	0
Total	12	6	4	8	36
Environment samples					
Contact surfaces with meat	43	nd	0	100 (3/3)	0
Not contact surfaces with meat	50	nd	50 (1/2)	50 (1/2)	50 (1/2)
Scalding water	0	nd	0	0	0
Total	37.5	nd	20	66.6	0

nd: not done

Table 7. Prevalence (%) of *Salmonella* per sample type and sampling day in *S4.*

	Prevalence (Pos/N)			
	Total	Sampling Day 1	Sampling Day 2	Sampling Day 3
Pig samples				
Lymph nodes	60	30 (3/10)	80 (4/5)	100 (5/5)
Colon content	45	0	100 (5/5)	80 (4/5)
Carcass sponge	35	0	60 (3/5)	80 (4/5)
Liver sponge	40	0	60 (3/5)	100 (5/5)
Tonsils	0	0	0	0
Total	36	6	60	72
Environment samples				
Contact surfaces with meat	50	nd	50 (1/2)	50 (1/2)
Not contact surfaces with meat	75	nd	100 (2/2)	50 (1/2)
Scalding water	50	nd	100 (1/1)	0
Total	60	nd	80	40

nd: not done

Table 8. Prevalence (%) of *Salmonella* per sample type and sampling day in *S5.*

	Prevalence (Pos/N)		
	Total	Sampling Day 1	Sampling Day 2
Pig samples			
Lymph nodes	0	0	0
Colon content	18.8	37.5 (3/8)	0
Carcass sponge	0	0	0
Liver sponge	0	0	0
Tonsils	0	0	0
Total	3.8	7.5	0
Environment samples			
Contact surfaces with meat	25	50 (1/2)	0
Not contact surfaces with meat	0	0	0
Scalding water	0	0	0
Total	10	20	0

nd: not done

Table 9. *Salmonella* serotypes distribution in pig and environmental samples (n.strains)

Serotype	n.	Pig samples				Environmental samples				
		Lymph nodes	Caecal content	Carcass sponge	Liver sponge	Deahiring equipment	Carcass splitter and knives	Drain water	Dirty zone walls	Scalding water
Derby	44	16	7	7	8	1	1	2	1	1
Typhimurium	24	2	8	3	1	1	4	5		
Panama	19		2	12	5					
Livingstone	5	5								
Infantis	5		5							
Rissen	4		1		3					
Newport	1	1								
Bredeney	1	1								
Total	103	25	23	22	17	2	5	7	1	1

Table 10. *Salmonella* serotypes distribution per slaughterhouse in pig samples.

Serotype	Tot	Slaughterhouse			
		S1	S3	S4	S5
Derby	40	5	2	33	
Typhimurium	14		6	1	7
Panama	19		19		
Livingstone	5	5			
Infantis	5		1	2	2
Rissen	4		4		
Newport	1			1	
Bredeney	1			1	
Total	89	10	32	38	9

Table 11. *Salmonella* serotypes distribution per slaughterhouse in environmental samples.

Serotype	Tot	Slaughterhouse		
		S3	S4	S5
Derby	4		4	
Typhimurium	10	8		2
Total	14	8	4	2

Table 12. Distribution of the *Salmonella* serotypes isolated in pig and environmental samples per slaughterhouse (S1 → S5) and sampling day (I → IV); the number of strains isolated is indicated between brackets.

SAMPLES	S1I	S1II	S3I	S3II	S3III	S3IV	S4I	S4II	S4III	S5I
Carcass						Panama (12) Typhimurium unt (3)		Derby (3)	Derby (4)	
Colon content			Infantis (1) Typhimurium 193 (1)			Panama (2) Rissen (1)		Derby (5)	Derby (2) Infantis(2)	Typhimurium 193 (7) Infantis (2)
Mesenteric lymph nodes	Derby (5)	Livingstone (5)	Typhimurium 193 (1)		Derby (2)		Bredeney (1) Newport (1) Typhimurium 193 (1)	Derby (4)	Derby (5)	
Liver				Typhimurium 104B (1)		Panama (5) Rissen (3)		Derby (3)	Derby (5)	
Drain water				S.typhimurium U302 (1)	Typhimurium 59 (1)	Typhimurium unt (3)		Derby (1)	Derby (1)	
Dirty zone wall								Derby (1)		
Splitting equipment					Typhimurium 59 (2)				Derby (1)	Typhimurium 193 (2)
Dehairing equipment					Typhimurium 59 (1)			Derby (1)		
Scalding water								Derby (1)		

Table 13. Summary of individual antimicrobial resistance frequency among 32 *Salmonella* serotypes.

		Number of isolates and (percentage) resistance to each antimicrobial							
SEROTYPE	**n.**	**A**	**C**	**S**	**Su**	**T**	**Tm**	**Ax**	**Kf**
Derby	8	6 (75)	4 (50)	0	6 (75)	6 (75)	6 (75)	2 (25)	8 (100)
Typhimurium	23	23 (100)	7 (30.5)	7 (30.5)	14 (61)	14 (61)	3 (13)	15 (65.2)	23 (100)
Rissen	1	0	0	0	0	1 (100)	0	0	1 (100)
Total	32	29 (90.7)	11 (34.4)	7 (22)	20 (62.5)	21 (65.7)	9 (28.2)	17 (53.1)	32 (100)

Table 14. Characteristics of the 103 strains of *Salmonella serotypes* (n. of isolates) .

SEROTYPE	Slaugtherhouse SD	PULSETYPE	PP (kb)	SOURCE	AMR PATTERN
Derby (4)	S1I	DerX21	180;90;3.6	Lymph node	ACSuTTmKf
Derby (1)		DerX23	90;3.6	Lymph node	fully sensitive
Derby (2)	S3III	DerX24	90;11.0;5.4	Lymph nodes	ASuTTmKfAx
Derby (2)	S4II	DerX22	12.0;6.2;5.7	Lymph nodes	Kf
Derby (7)	S4II e S4III	DerX22	5.7	Lymph nodes	fully sensitive
Derby (7)	S4II e S4III	DerX22	5.7	Colon content	fully sensitive
Derby (2)	S4II	DerX22	5.7;5.3;4.2	Liver	fully sensitive
Derby (6)	S4II e S4III	DerX22	5.7	Liver	fully sensitive
Derby (7)	S4II e S4III	DerX22	5.7	Carcass	fully sensitive
Derby (1)	S4II	DerX22	5.7	Scalding water	fully sensitive
Derby (2)	S4II e S4III	DerX22	5.7	Drain water	fully sensitive
Derby (1)	S4II	DerX22	5.7	Dirty zone wall	fully sensitive
Derby (1)	S4II	DerX22	5.7	Dehairing equipment	fully sensitive
Derby (1)	S4III	DerX22	5.7	Splitting equipment	fully sensitive
Typhimurium 193 (2)	S3I	STYMXB.0051	110;7.0	Colon content	ASSuTTmKf
Typhimurium 104B (1)	S3II	Tm120X15	11.0;6.5;5.8;4.3;3.8	Liver	ASSuTkf
Typhimurium U302 (1)		STYMXB.0058	120;5.2;4.7;4.4;3.7	Drain water	ACSuTTmKf
Typhimurium 59 (1)	S3III	STYMXB.0079 like	free	Dehairing equipment	ASSuTKf
Typhimurium 59 (1)		STYMXB.0079 like	free	Drain water	ASSuTKf
Typhimurium 59 (1)		STYMXB.0079 like	free	Splitting equipment	ASSuTKf
Typhimurium 59 (1)		STYMXB.0079 like	free	Dirty zone wall	ASSuTKf
Typhimurium unt (3)	S3IV	TmuntX16	130;6.7;4.1;3.6;3.3	Carcass	ACSuTKfAmc
Typhimurium unt (3)		TmuntX16	130;6.7;4.1;3.6;3.3	Drain water	ACSuTKfAx
Typhimurium 193 (1)	S4I	Tm193X13	250;90;3.2	Lymph nodes	fully sensitive
Typhimurium 193 (7)	S5I	STYMXB.0046	55	Colon content	AKfAx
Typhimurium 193 (2)		STYMXB.0046	60	Splitting equipment	AKfAx
Panama (12)	S3IV	PanX18	free	Carcass swabs	fully sensitive
Panama (2)		PanX18	free	Colon content	fully sensitive
Panama (5)		PanX18	free	Liver	fully sensitive
Livingstone (4)	S1II	LivX15	free	Lymph nodes	fully sensitive
Livingstone (1)		LivX15	85	Lymph nodes	fully sensitive
Infantis (1)	S3I	SINFXB.0074	free	Colon content	fully sensitive
Infantis (2)	S4III	SINFXB.0039	free	Colon content	fully sensitive
Infantis (2)	S5I	SINFXB.0014	free	Colon content	fully sensitive
Rissen (1)	S3IV	RisX10	4,8	Colon content	TKf
Rissen (3)		RisX9	free	Liver	fully sensitive
Newport (1)	S4I	NewX70	free	Lymph nodes	fully sensitive
Bredeney (1)		BreX16	free	Lymph nodes	fully sensitive

Table 15. Resistance genetic sequence identified in class 1 integron-carrying multidrug-resistant strains of *Salmonella*.

SEROTYPE	AMR pattern	n.	Int1	CS	aadA1	aadA2	bla(tem)	bla($carb_2$)	cat1	cat2	dhfr1	sul1	tetA(A)	tetA(B)	tetA(G)
S.derby	ACSuTTmKf	4	pos	pos			+	-	-	-	+	+	-	+	+
S.typhimurium DT 193	ASSuTTmKf	2	neg	neg	+	+	+	-			-	-	+	-	+
S.typhimurium DT104	ASSuTKf	1	neg	neg	+	+	+	-				-	-	+	+
S.typhimurium U302	ACSuTTmKf	1	pos	pos			-	-	+	-	-	+	-	+	+
S.derby	ASuTTmKfAx	2	neg	neg			+	-			-	-	-	+	+
S.typhimurium DT59	ASSuTKf	4	neg	neg	+	+	+	-				-	-	+	+
S.typhimurium unt	ACSuTKfAx	6	pos	pos			-	-	+	-	-	-	-	+	+
S.rissen	Tkf	1	neg	neg			-	-				-	+	-	+
S.typhimurium DT 193	AKfAx	9	neg	neg			+	-							

Figure 1. *Salmonella* prevalence in pig samples per slaughterhouse.

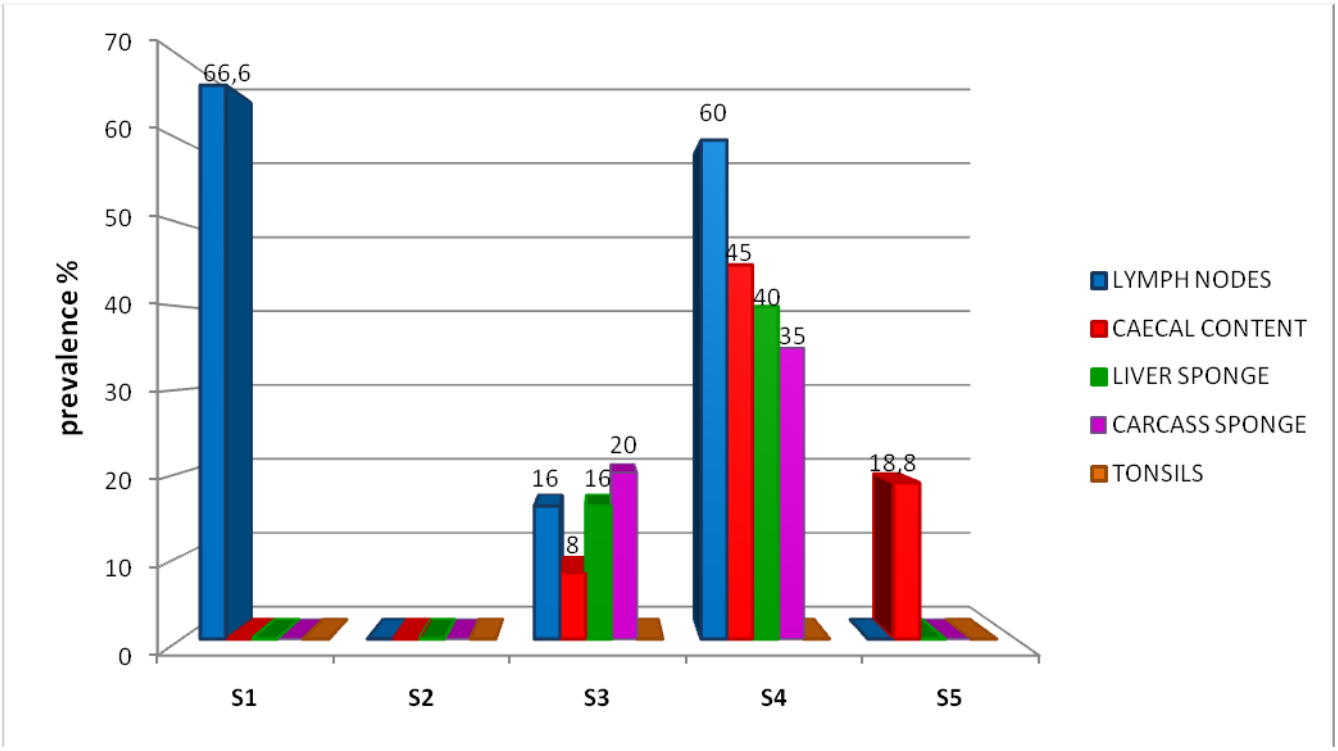


Figure 2. *Salmonella* prevalence in environmental samples per slaughterhouse.

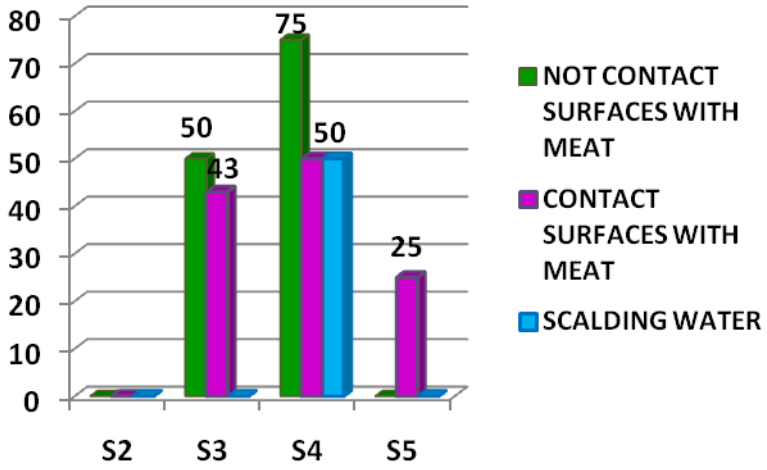


Figure 3. Prevalence of *Salmonella* serotypes isolates from pig and environmental samples.

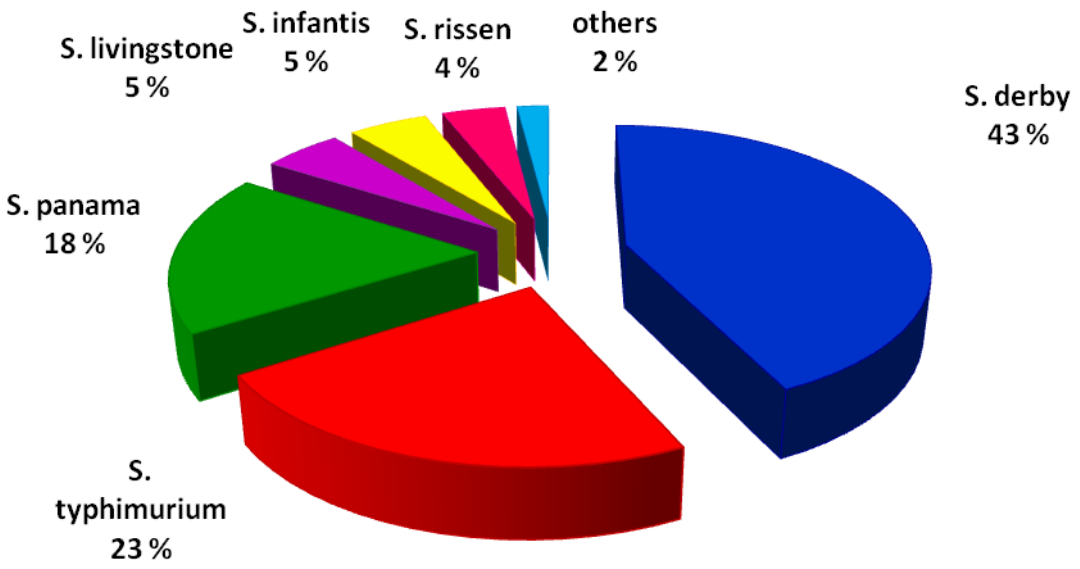


Figure 4. Prevalence of *S. typhimurium* phage types (n. of strains)

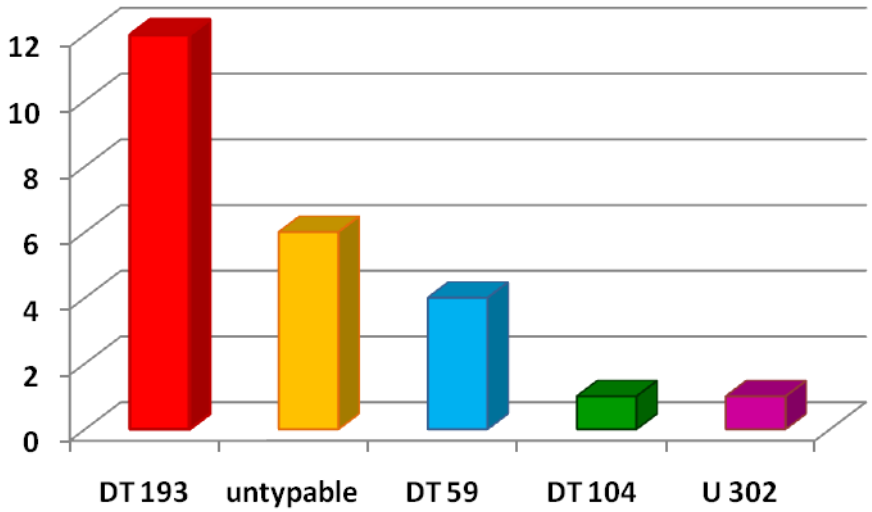
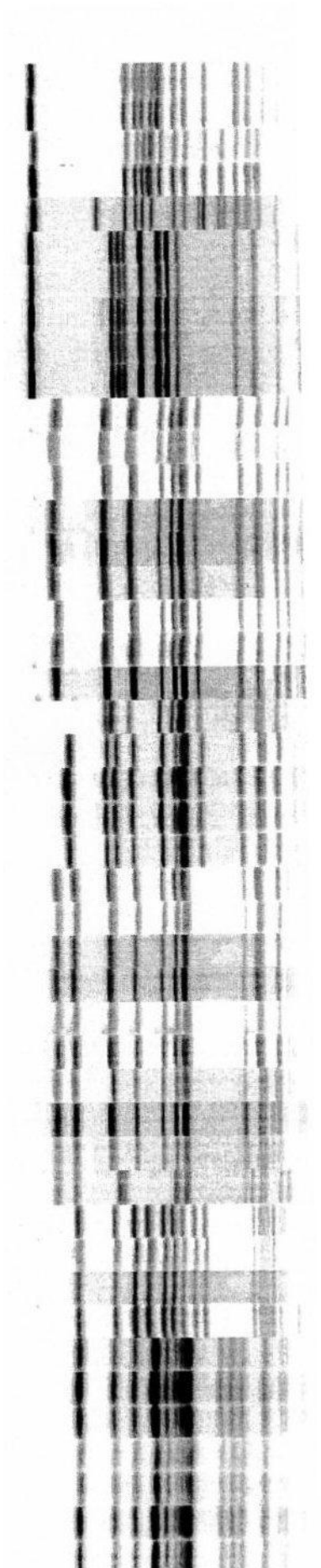
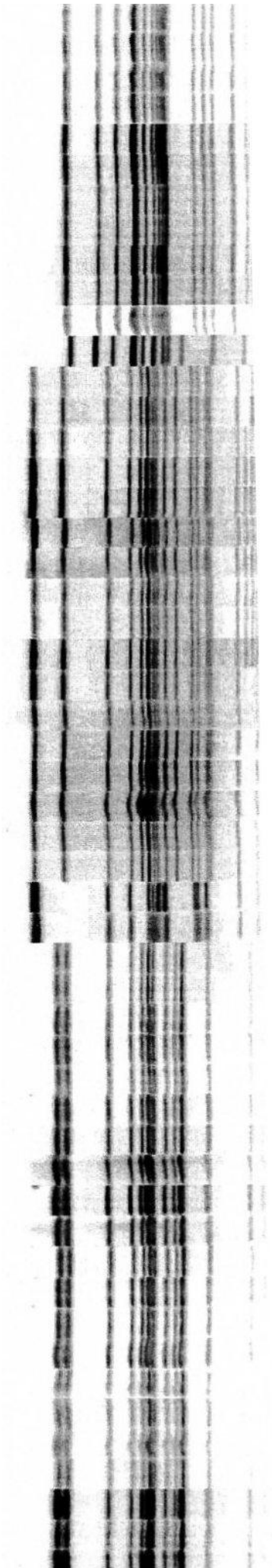


Figure 5. PFGE fingerprints of *Salmonella* isolates, in relation to the serotype, pulsetype, slaughterhouse, sampling day (SD) and source of the strains.



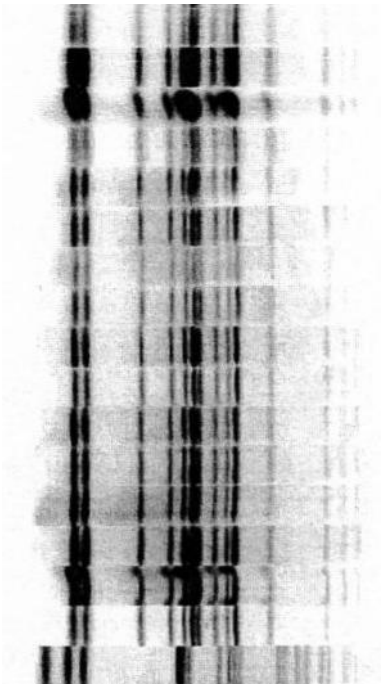
SEROTYPE	PULSE-TYPE	SLAUGHTERH. AND SD	SOURCE
Infantis	SINFXB.0014	S5I	Colon content
Infantis	SINFXB.0014		Colon content
Infantis	SINFXB.0039	S4I	Colon content
Infantis	SINFXB. 0039		Colon content
Infantis	SINFXB.0074	S3I	Colon content
Livingstone	LivX15		Lymph node
Livingstone	LivX15		Lymph node
Livingstone	LivX15	S1II	Lymph node
Livingstone	LivX15		Lymph node
Livingstone	LivX15		Lymph node
Typhimurium 193	STYMXB.0046		Colon content
Typhimurium 193	STYMXB.0046		Colon content
Typhimurium 193	STYMXB.0046		Colon content
Typhimurium 193	STYMXB.0046		Colon content
Typhimurium 193	STYMXB.0046	S5I	Colon content
Typhimurium 193	STYMXB.0046		Colon content
Typhimurium 193	STYMXB.0046		Splitting equipment
Typhimurium 193	STYMXB.0046		Colon content
Typhimurium 193	STYMXB.0046		Splitting equipment
Typhimurium 104B	Tm120X15	S3I	Liver
Typhimurium 59	STYMXB.0079like		Drain water
Typhimurium 59	STYMXB.0079like	S3III	Splitting equipment
Typhimurium 59	STYMXB.0079like		Dehairing equipment
Typhimurium 59	STYMXB.0079like		Dirty zone wall
Typhimurium unt	Tmunt16		Carcass
Typhimurium unt	Tmunt16		Carcass
Typhimurium unt	Tmunt16	S3IV	Carcass
Typhimurium unt	Tmunt16		Drain water
Typhimurium unt	Tmunt16		Drain water
Typhimurium unt	Tmunt16		Drain water
Typhimurium U302	STYMXB.0058	S3II	Drain water
Typhimurium 193	STYMXB.0051	S3I	Colon content
Typhimurium 193	STYMXB.0051		Colon content
Typhimurium 193	Tm193X13	S4I	Lymph node
Rissen	RisX9		Liver
Rissen	RisX9		Liver
Rissen	RisX9		Liver
Rissen	RisX10		Colon content
Panama	PanX18	S3IV	Carcass
Panama	PanX18		Carcass
Panama	PanX18		Carcass
Panama	PanX18		Carcass
Panama	PanX18		Carcass

Figure 5 (continued)



Panama	PanX18		Carcass
Panama	PanX18		Carcass
Panama	PanX18		Carcass
Panama	PanX18		Carcass
Panama	PanX18		Carcass
Panama	PanX18	S3IV	Colon content
Panama	PanX18		Colon content
Panama	PanX18		Liver
Panama	PanX18		Liver
Panama	PanX18		Liver
Panama	PanX18		Liver
Panama	PanX18		Liver
Bredeney	BreX16	S4I	Lymph node
Derby	DerX23		Lymph node
Derby	DerX23		Lymph node
Derby	DerX21		Lymph node
Derby	DerX21		Lymph node
Derby	DerX21		Lymph node
Derby	DerX21		Lymph node
Derby	DerX21		Lymph node
Derby	DerX21		Lymph node
Derby	DerX21	S1I	Lymph node
Derby	DerX21		Lymph node
Derby	DerX23		Lymph node
Derby	DerX23		Lymph node
Derby	DerX23		Lymph node
Derby	DerX23		Lymph node
Derby	DerX23		Lymph node
Derby	DerX23		Lymph node
Derby	DerX23		Lymph node
Derby	DerX24	S3III	Lymph node
Derby	DerX24		Lymph node
Derby	DerX22		Dehairing equipment
Derby	DerX22		Liver
Derby	DerX22		Liver
Derby	DerX22	S4II	Liver
Derby	DerX22		Carcass
Derby	DerX22		Carcass
Derby	DerX22		Carcass
Derby	DerX22		Colon content
Derby	DerX22		Colon content
Derby	DerX22		Carcass
Derby	DerX22	S4III	Carcass
Derby	DerX22		Carcass
Derby	DerX22		Carcass
Derby	DerX22		Lymph node

Figure 5 (continued)



Derby	DerX22		Liver
Derby	DerX22		Liver
Derby	DerX22	S4III	Liver
Derby	DerX22		Splitting equipment
Derby	DerX22		Drain water
Derby	DerX22		Lymph node
Derby	DerX22		Lymph node
Derby	DerX22		Colon content
Derby	DerX22		Colon content
Derby	DerX22		Colon content
Derby	DerX22	S4II	Colon content
Derby	DerX22		Colon content
Derby	DerX22		Scalding water
Derby	DerX22		Drain water
Derby	DerX22		Dirty zone wall
Derby	DerX22		Liver
Newport	NewX70	S4I	Lymph node

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INDEX